A ^{1}H NMR metabolomic study of the metabolic effects of pesticides on the non-target invertebrates the blue mussel *Mytilus edulis* and the non-biting midge larvae *Chironomus riparius*

By

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Abstract

Metabolomics is a technique that can be used to investigate the metabolic profiles of an organism by measuring a large number of the low molecular weight metabolites in the metabolic pool. ^{1}H NMR spectroscopy is an analytical tool that is unbiased, and a non-destructive way of investigating metabolic perturbations in organisms exposed to environmental stress.

In this study two model organisms, *Mytilus edulis* and *Chironomus riparius* were used to investigate the effects of several pesticides: lindane and atrazine in the mussels, and fenitrothion, methiocarb and permethrin in the midge larvae. The impact of hypoxia and starvation were also investigated alongside the exposure to atrazine in the mussel. The pesticides used in the mussel study have different modes of action, but produce similar changes to behaviour and can cause starvation and mild hypoxia. Acetonitrile/$^{2}H_2O$ (60/40 % v/v) extracts of foot muscle of mussels subjected to hypoxia, or starvation, or to low or high doses of pesticide were analysed using ^{1}H NMR spectroscopy to produce metabolic profiles associated with these treatments. Discriminant analysis showed significant differences between treated and control animals and gave a clear separation between all treatment groups. Atrazine profiles were clearly separated from the starved and hypoxic animals and the animals exposed to high and low doses were also separated. Lindane treatment was separated from control animals in a dose-dependent way. This was associated with an increase in alanine concentrations and a decrease in all other identified metabolites. The study of midge larvae used the same approach, but using extracts of pooled whole body homogenates (10 animals

per sample) instead of tissue from individuals. The animals were subjected to low, environmentally relevant dose levels of three pesticides; fenitrothion, methiocarb and permethrin. The first two of these have a similar mode of action, inhibition of acetylcholine esterase, whilst the latter is an axonal poison acting on cation channels. The metabolic profiles associated with these treatments showed a clear separation between all treatment groups and between treated and control animals. Fenitrothion treatment was associated with an increase in alanine (on average of 93.3 μM relative to controls) and lactate concentrations compared with controls and other treatments. Methiocarb caused a reduction in arginine, leucine and lysine concentrations to half of the control level. Permethrin produced a reduction in tyrosine and phenylalanine concentrations to half that of the control group. In both these experiments the use of ^{1}H NMR metabolomics enabled the separation the effects of all of the treatments and stressors from each other and from the controls. It demonstrates the potential of the metabolomic approach to provide separation of the effects of poisoning from those of environmental stress, and to distinguish between toxicants with similar modes of action.

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Abbreviations

^{1}H NMR	Proton Nuclear Magnetic Resonance
$^{2}H_2O$	Deuterated water
ACD/Labs	Advanced Chemistry Development Laboratories
AchE	Acetylcholine esterase
ACN	Acetonitrile
ANOVA	Analysis of Variance
bp	Base Pairs
cHSQC	Carbon Heteronuclear Single Quantum Coherence
COSY	Correlation Spectroscopy
DNA	DeoxyriboNucleic Acid
dNTP	DeoxyriboNucleotide TriPhosphate
EDTA	EthyleneDiamineTetraacetic Acid
EtBr	Ethidium Bromide
fid	Free Induction decay
FT	Fourier Transformation
GABA	Gamma-AminoButyric Acid
GC/MS	Gas Chromatography/Mass Spectrometry
GC/TOF-MS	Gas Chromatography/Time of Flight-Mass Spectrometry
GRI Ltd	Genetic Research Instrumentation Limited
HPLC	High Performance Liquid Chromatography
KH_2PO_4	Potassium dihydrogen phosphate
LDA	Linear Discriminant Analysis
$MgCl_2$	Magnesium Chloride
μM	Micro Molar
Na_2HPO_4	Sodium Phosphate
NaAc	Sodium Acrylate
NOAEL	No Observable Adverse Effect Level
NOLL	No Observed Lethality Level
OP	Organophosphate
ppb	Parts Per Billion
ppm	Part Per Million
PC	Principal Component
PCA	Principal Components Analysis
PCR	Polymerase Chain Reaction
RNA	RiboNucleic Acid
RNAse	RiboNucleic Acid Esterase
Satfrq	Saturation Frequency
Satpwr	Saturation Power
Satdly	Saturation Delay
SDS	Sodium Dodecyl Sulfate
SPE	Solid Phase Extraction
TBE	Tris base/Boric acid/EDTA
TMSP	Sodium 3-(trimethylsilyl)-2,2,3,3-d_4-propionate
TOCSY	Total Correlation Spectroscopy
Tris	Tris(hydroxymethyl)aminomethane

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Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research reward. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

Chapter 1

Introduction

All animals are faced with the problem of foreign organic molecules entering the metabolic pool, mainly from food. They have a set of excretory mechanisms that enable them to remove those compounds from the metabolic pool in order to prevent disruption of metabolism. Detoxification mechanisms that either reduce the pharmacological activity of the potentially poisonous compounds and/or speed up their elimination from the metabolic pool are present in animals. However, the biochemical systems present vary between species, and between individuals within a species, leading to a range of tolerances and differential toxicity of compounds between groups of animals. Not all toxicants are of anthropogenic origin, but since the time of the industrial revolution over 150 years ago, a wide range of compounds have been introduced into the environment, and some of these have been produced in large quantities. Some of these substances can have deleterious effects on organisms, at individual, population and ecosystem levels. Aquatic organisms can be particularly vulnerable when they ventilate their respiratory surface with water. The ventillatory mechanisms of these organisms have a short diffusion distance between the external medium and the body fluids, and a high circulation rate of body fluids under the respiratory surface to match a flow of external medium across the outer surface of the gill. This can lead to high uptake rates, especially for small non-polar molecules. A combination of this large potential for accumulating foreign organic molecules, and the extensive use of aquatic environments to remove pollutants from human populated areas (Rand, 2003) exposes aquatic animals at risk. Some chemicals such as pesticides are deliberately introduced into the environment in a controlled way to control pests (for instance, insects that compete with

man for resources, or act as vectors of diseases). Since the introduction of agricultural pesticides over the last 50 years some pollution incidents have resulted, usually through their misuse or inappropriate disposal. Although the impact of pollutants can be dramatic and obvious as exemplified by large fish kills resulting from a large scale spillage of some toxicants, there can be more subtle effects that are not readily discernable, for instance reductions in scope for growth or impairment of reproductive capacity during long term exposures to low levels of contaminants. Although tighter regulations have reduced such large scale incidents there is still a continuous flow of chemicals leaching from the land into river systems and ground water and there is in many cases uncertainty concerning the level of damage that might be caused to the ecosystem.

Legislation is in place in many parts of the world to control the quality of drinking water, and this has vastly reduced the risk of human ingestion of toxic substances from this source. Legislation such as the European Union Water Framework Directive and it daughter directive (the Ground Water Directive), and equivalent legislation in countries such as USA, Canada, and Australia have led to significant improvements in the quality of environmental waters. However, a wide range of anthropogenic compounds, such as pesticides, industrial chemical, personal care products, household care products, and pharmaceuticals still reach environmental waters. Some of these compounds have been designed to have specific biological activity, and may have deleterious effects on both the target organism, and on many non-target species, including humans. These pollutants can be absorbed directly into the

bodies of aquatic organisms such as fish and invertebrates as they remove oxygen from the water for respiration (De Zwaan *et al*. 1996). This occurs as a passive process due to differences in the concentration gradient inside and outside of the organism. This is an additional route of entry compared with terrestrial species where the main route of entry is through the digestive tract following ingestion of contaminated food and water. Therefore it is important that we understand the effects these pollutants have on vulnerable organisms such as those in the aquatic environment.

The toxicity of many compounds has been assessed because of regulatory requirements. This applies particularly to pesticides where a full toxicology package (mammals, fish and a range of invertebrates and plants) and environmental fate studies are required for registration. More recently environmental risk assessments have been required for pharmaceutical compounds for both medical and veterinary uses. The new REACH legislation that is currently being phased in requires a risk assessment for industrial chemicals that are produced and/or used in significant quantities. However, for a large number of industrial compounds there is no toxicological information available. The effects of old compounds of particular concern (such as polyaromatic hydrocarbons, aestrogens, detergents) and in some cases their metabolites, have been extensively studied but with a relatively small number of methods and of target organisms (Page et al., 1998).Many of these are acute toxicity tests that involve the addition of a toxic insult to the environment of the organism to be studied over a short exposure time (normally between 1-7 days exposure). The toxicants are applied at a range of

concentrations, and predefined symptoms are recorded at a range of times or just at the end of the assay (Girling et al., 2000). The symptoms can be changes in behaviour, growth and activity, but the most commonly used endpoint for such studies (for both vertebrates and invertebrates) is death (Rand, 2003). These tests provide a measure of toxicity that is usually expressed as the median concentration (that produces the symptom of toxicity in 50% of the population), but they do not provide information on the mode(s) of action. Effects of lower concentrations of these substances are also not detected. Chronic toxicity studies where exposure of the organism to a toxicant is over a longer time period are conducted, but are not used as often as acute assays since the former are time consuming and expensive, and can involve observations over the whole life cycle.

Toxicity results from disruption of the metabolism of the organism. However, the toxicological symptoms that are observed may not be as a direct result of a primary lesion (e.g., inhibition of an enzyme such as acetyl cholinesterase in the nervous system, or blocking an ion channel in a muscle cell) but may be due to a series of secondary changes that are caused by the primary lesion. In order to understand the basis of observed toxicological symptoms then it is necessary to observe changes in metabolism. These can be studied at a number of levels, using a wide range of analytical methods including enzyme assay systems, gas or liquid chromatography linked to a number of detectors (including mass spectrometers), and in recent times nuclear magnetic resonance spectroscopy (NMR). The linkage of these techniques with

multivariate data analysis has given rise to the fields of metabolomics/metabonomics.

This study aimed to use metabolomic methods to study the impact of a range of pesicides on metabolism of some aquatic invertebrate species. High-resolution NMR spectroscopy was chosen as the analytical method to be used to help to gain understanding of biochemical pathways modified by exposure to toxicants.

1.1. Introduction to NMR spectroscopy

Since the discovery of the phenomenon of NMR spectroscopy in 1945 it has developed to become a powerful analytical tool. Since NMR is the only method for elucidating the molecular structure of a compound in solution its application has spread through to chemistry, biology and even medical diagnosis. It is the magnetic property or “spin” of the nucleus of an atom that is the basis for NMR spectroscopy. There are many texts that provide a comprehensive explanation of the theory and application of NMR (Berger, 2004, Breitmaier, 1993, Freeman, 1998, Freibolin, 1991, Gunther, 1998). In brief, when a compound is in a strong magnetic field, it is possible to transfer energy into the spin system by using a particular radio frequency pulse (Larmor frequency). The system will then relax back to a state of equilibrium after the pulse has ceased, producing a weak signal (chemical shift) that can be detected. Since the spin of each nucleus is also affected by the magnetic fields of the neighbouring molecules, it is therefore possible to separate

signals from different atomic surroundings. From such signals it is then possible to determine the structure of the molecule. A choice of nuclei for study can be achieved by changing the frequency of the pulse so that it is in resonance with the particular spin (Freeman, 1998). The most frequently used nuclei that can be detected for the study of organic molecules are ^{1}H, ^{13}C, ^{15}N, ^{19}F and ^{31}P, with the proton being the most sensitive.

One aspect of NMR that has limited its use has been its lack of sensitivity, with the loss of signal due to background noise. Recent advances within the last fifteen years, however, have led to much higher resolution instruments. This has been due to the development of stronger superconducting magnetic fields coupled with advances in computer technology. These magnetic fields are produced by coils of superconducting wire being bathed in liquid helium at a temperature of 4.2 K, producing a magnetic field strength of 5.17 T, corresponding to 220 MHz for proton NMR (Lindon and Nicholson, 1997). The sensitivity of the NMR instrument is directly proportional to 2/3 of the power of this field strength, and there have been increases in these superconducting fields in recent years. Most modern instruments operate at 600-800 MHz and some even up to 1 GHz (Reo, 2002). Along side this improvement there have been developments in the production of much shorter, more complex, intense pulse sequences, that have revolutionised the application of NMR by shortening acquisition times (time needed to complete a scan of the sample and acquire a spectrum).

These high field machines are used extensively to model the three-dimensional structures of macromolecules. They are also used to obtain one- or two-dimensional spectra leading to the identification of specific molecules within complex mixtures (Fan, 1996). This improved sensitivity has also led to novel applications in the field of toxicology. Pharmaceutical chemists use NMR methods to confirm the structures of both synthetic and natural products being considered for use in drug production. These methods can also be used to analyse the biological fate of new products by investigating metabolites of drugs in body fluids. These and many other applications within this field are beyond this review and can be found in a review by (Holzgrabe et al., 1998).

1.2. Use of metabolomics

One application that has arisen from the genome sequencing era is that of metabolomics (metabonomics), the simultaneous measurement of low molecular weight metabolites within a tissue or biofluid sample. The technique was pioneered by J. K. Nicholson of Imperial College London in the 1980's utilizing the analytical technique of high-resolution NMR spectroscopy (Reo, 2002). The metabolome is a highly complex mixture of biochemicals, with the main metabolites consisting of organic acids, amino acids, sugars, sugar phosphates, fatty acids, and steroids, all of which are of interest in defining an organism's physiology (Goodacre *et al*., 2004). Previous investigations into metabolites traditionally used techniques such as mass spectrometry coupled with gas chromatography (GC) or high performance liquid chromatography (HPLC). These chromatographic techniques were initially found to be time

consuming and can involve extensive sample preparation that can interfere with the metabolic profile (Lindon *et al*., 2004). Liquid Chromatography (LC/MS) methods for instance may require a pre- or post column derivatization of analytes, whereas with Gas Chromatography (GC/MS) analysis sample derivatization is almost mandatory as the majority of the metabolites of interest are non-volatile in their original composition (Liebeke *et al*., 2010). Although this requirement for the modification of non-volatile compounds maybe used to an advantage by exploiting selective chemical enrichment and fractionation for the profiling of trace compounds in the presence of bulk metabolites (Birkemeyer et al., 2003). The very nature of the ionisation of the samples during mass spectral analysis leads to the destruction of the original sample. In contrast high resolution NMR spectroscopy uses only radio frequency radiation and is therefore a non-destructive technique where the sample can be reused or analysed. Also the profile of metabolites can be observed with no pre-selection of measurement parameters that maybe required for the other techniques as well as being able to observe non-reactive metabolites not detectable by MS (Dunn and Ellis, 2005). Furthermore the reduction of sample preparation steps for NMR based metabolic profiling can increase throughput and lower per sample costs. Although recent studies are now looking to improve and speed up preparation times for the derivatisation steps with Liebeke's group using a microwave irradiation assisted method to reduce the silylation step from 120 minutes to just 6 minutes (Liebeke et al., 2010). Such improvements to all these analytical methods make the choice of format for a particular study difficult,

now often several techniques, when available, are used in combination (Wu et al., 2008, Lane et al., 2008, Wishart et al., 2007)

The metabolome of particular species of interest can vary greatly in size and complexity. The human metabolome contains over 7900 identified metabolites including both water-soluble and lipid soluble compounds (Wishart et al., 2007), whilst plant metabolomes are estimated to exceed 200 000 metabolites (Fiehn, 2002). Therefore the identification of the entire metabolic profile on a per sample bases would be impossible, with differing extraction as well analytical techniques needing to be employed. A typical one dimensional (1D) NMR method would routinely detect < 150 metabolites pertaining to < 1 % of the metabolome of the species investigated with many of these remaining unidentified (Wishart, 2008). This highlights the limitations of NMR-based metabolomic techniques due to its relatively low sensitivity, with a lower limit of detection quoted at 1-5 µM in ~ 500 µL of sample (Wishart, 2008). Although higher field magnets (≥ 900 MHz) and cryogenically cooled probes are able to reduce these limitations this type of equipment is expensive, so therefore limited to specialist laboratories. The use of GC/MS has been used to detect > 300 metabolites in the freshwater amphipod *Diporeia* spp, although only 4 % of these were positively identified (Ralston-Hooper et al., 2008), but this type of equipment is more readily available.

The format for this study was ^{1}H NMR and was chosen as the equipment was available and had a cryoprobe installed increasing sensitivity. Also as the amount of dosed tissue samples would also be limited and are labour

extensive and time consuming, the fact that this technique does not destroy the samples would allow further study to be conducted on the samples without the need to reproduce the toxicological process. The tissues themselves are not analysed directly, therefore sample preparation steps are also important. Since blood or coelomic fluid are involved in the mass transport of substances from sites of absorption or production to target tissues, these are ideal for metabolic investigations, although these were not used here. It is possible to use untreated samples of tissues when the test organism is very small, or subsamples of specific tissues, where removal of the sample matrix from the metabolites is needed. It is also usually necessary to remove macromolecules, such as proteins, from the sample to reduce large overlapping peaks in the resultant spectra (Viant et al., 2009, Wu et al., 2008, Tiziani et al., 2008, Lin et al., 2007, Lin et al., 2006). There are several methods of achieving this. Ultra-filtration devices that use a 10 kDa cut off point during centrifugation have been employed. Other methods of removing macromolecules include the use of organic solvents (e.g., acetone and acetonitrile) that precipitate proteins. Both of these methods are relatively simple to use and have been extensively reviewed (Viant, 2003, Viant, 2007b, Wu et al., 2008).

Using these techniques it is possible to obtain useful spectra that reflect the array of small molecules in the metabolic pool of an organism. Where changes occur due to a toxic insult, these can be characterised and can aid in the interpretation of the biochemical consequences of the interaction of the toxicant with the biological system. This is because an insult of a stressor may

cause changes in the concentrations of endogenous metabolites through reactions with different biochemical pathways within the organism so as to maintain homeostasis. It is these concentration changes that metabolomics is able to detect and can be used as a fingerprint or biomarker of toxicity. So even at low concentrations of a particular toxin where homeostasis is still maintained the cellular composition of metabolites will be altered and be present within these biofluids or tissue extracts. As the spectra of such fluids contain vast quantities of endogenous molecules, produced from the many different biochemical pathways, this can lead to highly complex spectra. To acquire any meaningful information from such data the natural biological variation within a set of samples will need to be identified (Lenz *et al*., 2003). Such variations can be due to genetic differences within a species with the expression of different enzyme concentrations, or to more simple changes caused by for instance a change of diet (Solanky *et al*., 2003). Therefore it is critical to a study that a comprehensive understanding of the 'normal' physiological variation within a species is obtained. In order to achieve this it is necessary to analyse many control specimens, and this will produce a large quantity of data that has to be interpreted (Saude *et al*., 2007).

1.3. Data reduction and pattern recognition

The large sets of data obtained need to be reduced in order to make it possible to interpret them. In order to achieve this, data sets are generally segmented into between 128-256 bins of data containing chemical shift regions of around 0.04 ppm (Anderson *et al*., 2008, Constaninou *et al*., 2004,

Davis *et al*., 2007, De Meyer *et al*., 2008, Parsons *et al*., 2009, Potts *et al*., 2001). This can be achieved with the use of software packages such as AMIX (analysis of mixtures). The data in the form of integrated peaks within the bins can then be handled using a spreadsheet such those available in Excel (Microsoft®) for analysis. The variations between the sets of spectra can still be extremely difficult to detect, especially by eye, and further analysis is needed to visualise the reduced spectral data. One such method is that of principle component analysis (PCA), an unsupervised method requiring no prior knowledge of the data set and acts to reduce the dimensionality of multivariate data sets whilst preserving most of the variance within it (Halouska and Powers, 2006).

PCA was first described by Karl Pearson in 1901, and is used to analyse a large number of variables to produce indices that are uncorrelated (orthogonal) and in order of their importance (the proportion of the total variation accounted for a particular component). Where variables are correlated there will be less non-zero principal components (PCs) than the original variables. In this way the large number of original, correlated variables is reduced to a smaller number of uncorrelated variables that describe fractions of the information in the original data set. Interpretation of the PCs is aided by examining the correlations of the original variables with the scores of the variables on the PCs. Those correlations are termed the loading of the variables on the PCs. The PCA does not change the relative positions of the original observations in the multidimensional space occupied by the data.

The result of the PCA is that the data points in n-dimensional space, where n equals the number of variables (256 bins), are reduced into a few principal components (PC's) that display the maximum variation within the data. Two-dimensional plots of the scores on the first few PCs show the scatter of the original variables in those two dimensions. When the treatments to which the individual scores were subjected are identified, then any patterns are easy to see. Interpretation of the plots involves identification of the PCs that separate the various treatments, and then using the loadings on those PCs to interpret them in terms of their biochemical significance. In some cases it may be necessary to use more than two PC's to achieve a clear separation. Any outliers will be easy to identify in the plots (Holmes *et al*., 1998, Solanky *et al*., 2003).

Partial Least Squares-Discriminant Analysis (PLS-DA) in contrast to PCA is a supervised method utilising the input of prior knowledge about the data set (e.g. dose concentration). PLS-DA determines a set of latent variables similar to principal components in a PCA, but indicating possible covariance between two matrices (scores) (Lindon et al., 2001). The output of this is the score matrix that can be plotted similarly as in PCA, with a predictor matrix, which estimates class affiliation, with a comparison of all vectors performed a percent of correct classifications can be obtained obtained (Wagner et al., 2006).

Discriminant analysis (DA) is another method that can be applied in the analysis of metabolomic data. The problem addressed by this method is the

separation of groups of observations on the basis of a set of variables. The solution to this problem was published independently by three different workers (Fisher, 1936, Hotelling, 1936, Mahalanobis, 1936). This technique optimises the separation between identified groups of observations on the basis of a set of variables that can be a set of scores on PCs or original variables (e.g., concentrations of various metabolites, or measures of masses of the various metabolites) that have been measured in all of the treatment groups. DA provides a series of discriminant functions (depending on the number of groups to be separated). These can be plotted, as for the PCs, to reveal the degree of separation of the groups and their relative positions. The discriminant functions can be used to predict the group membership of observations where this is not known. The functions can be interpreted in terms of the original variables in a way similar to that used for the PC's by examining the loadings of the original variables on the discriminant functions.

Due to the nature of the multivariate analyses used to identify patterns of variation between treatment groups it is essential to assess the quality of these supervised analysis techniques. This can be achieved by utilizing permutation testing where the Y matrix of treatment labels is randomised and misclassification error rates are calculated (Eriksson et al., 2001). Permutation tests use the assumption of the possibility that all of the treatment groups are equivalent and that every observation is the same before sampling begins. From this, a statistic can be calculated as to what extent the observation is unique by showing how likely it would be if the treatment assignments had been randomised. Cross validation, a method validating a predictive model,

can be used where a single observation is left out and a new test set produced. These new observations can then be compared to the original data set to show how probable that this observation is due to chance alone, replacing simple visual observations with robust statistics.

1.4. Applications of metabolomics

1.4.1. Clinical studies

The original applications of metabolomics by J. K. Nicholson (Nicholson *et al*., 1995, Nicholson, 1999) and others used proton NMR to investigate the small molecular weight substances in body fluids such as urine and plasma. The aim was to use biochemical profiling to provide fingerprints, based on the pattern of metabolites, of disease states (Warne *et al.*, 2000). Since then improvements in NMR sensitivity and the use of pattern recognition techniques for analysing the spectra has meant that there has been an increase in activity within this field. Interest from pharmaceutical companies into the early detection of drug-induced lesions has supported work to identify novel biomarkers of toxicity that can provide information at an early stage in the drug development process. An example is provided by the use of metabolomic studies to characterise renal toxicity using NMR spectra of urine (Holmes *et al*., 1998). This group used PCA techniques to analyse NMR spectra of rat urine, and were able to demonstrate a relationship between metabolite composition and the site of toxicity. However, the quantification of

these metabolites is time consuming and requires prior assumptions of importance of some metabolites to indicate a toxic effect. This can result in other low level changes being overlooked. There is, however, a possibility that some of these apparently minor metabolites may be more important than the larger fractions for providing understanding of the mechanisms of toxicity. In order to overcome these problems, automated data reduction procedures were developed. In these the spectrum is divided into equal regions of chemical shift ranges (bins) (Holmes *et al*., 1998, Webb-Robertson *et al*., 2005), and these are analysed without any prior assumptions to identify those regions of the spectra that differ between the treated and control individuals. The technique has been used on human urine samples from patients with inborn errors of metabolism (Constaninou *et al*., 2004). However, there are potentially problems when comparing the affected subjects with controls since there is significant inter-subject variability, due to for instance simple dietary differences. This problem is especially marked when analysing urine, and is less pronounced in plasma samples (Lenz *et al*., 2003).These studies highlighted the need to record the normal range of physiological variation.

The success of this investigation encouraged further studies of renal toxicity to classify the sites of lesions of a group of toxins. It was discovered that each of the 15 toxicants investigated was associated with a unique metabolic profile, and the onset of biochemical change was demonstrated to be toxin dependent. For most of the test compounds the site of toxicity were found to be predominantly in the nephron region. The team concluded that the

automated data reduction and PCA of spectra obtained for urine did allow the identification of specific biomarkers of toxicity (Holmes *et al*., 1998).

The success of studies such as these on the evaluation of xenobiotic toxicity has led to the formation of the Consortium for Metabonomic Toxicology (COMET) consisting of six pharmaceutical companies and the Imperial College of Science, London. The group was formed to define sets of methodologies of metabonomic data generation based on blood, serum and urine for toxicological screening of candidate drugs (Lindon *et al.*, 2003). The group continues to add results from studies using a range of model toxins to an electronic database that serves as a basis for modelling to predict toxicity. Initial investigations of hepatotoxicity and renal toxicity were based on rodent (rat and mouse) models. A comparison of the results from the set of methodologies between the six companies has been found to have a high degree of reproducibility and robustness. This has enhanced confidence in the reliability of this methodology. The next step is the production of an expert system to detect the toxic effects of xenobiotics based on combinations of biomarkers and changes in metabolite profiles measured using NMR techniques.

NMR spectra will display peaks resulting directly from the toxin and its metabolites, and those need to be removed before pattern recognition analysis so that they are not interpreted as resulting from a change in metabolism. In order to handle in a cost effective way the large number of spectra being produced, it was necessary to automate the method of removal

of the unwanted information. This was achieved by the development of software that removes and replaces those regions in the spectra whilst allowing the other regions to be retained for analysis. The aim is to achieve an NMR-based expert system that will be suitable for use in high-throughput *in vivo* screening programmes. This work has generated many new biomarkers of drug toxicity to further the understanding of mechanisms of toxicity and of biochemical effects of novel drugs (Lindon *et al*., 2003).

1.4.2. Environmental studies (terrestrial)

The large advances in the application of metabolomic methods in medical and veterinary areas have supported the application of these techniques in a wide range of organisms including micro-organisms, fungi, higher plants, and invertebrates, and in a range of areas of application. The latter include studies of wine quality, the impact of genetic manipulation on metabolism, and the impacts of pesticides on aquatic organisms. There have been advances in the analytical tools (e.g., such as NMR, LC-MS, GC-MS, capillary electrophoresis-MS) available for use in metabolomic studies. There is less pressure to develop high throughput screening of the type described above for use in the pharmaceutical area. However, the other fields have made significant progress in developing methodologies that can provide metabolomic information in a cost effective way. (Viant, 2003) states that apart from the initial costs of purchasing the NMR spectrometer, the per sample costs are relatively low compared to other instrumental analytical techniques.

There is a demand for the identification of novel biochemical biomarkers in a range of non-target species affected by environmental pollutants. This has traditionally involved measuring the residual level of bio-accumulated pollutants within exposed organisms, and relating this to specific behavioural or biochemical changes. With the introduction of metabolomics a more diagnostic approach to the changes in endogenous metabolites is now possible. Such investigations may be able to identify novel biomarkers that indicate exposure to low level exposures that produce sub-lethal, but possibly important effects that can cause long-term harm. Such sensitive biomarkers may allow the detection of exposure at an early stage so that remedial measures can be put in place before serious damage is caused at population levels.

One area of study has concentrated on the use of earthworms since these are of great importance in maintaining soil quality. These annelids accumulate metals and other contaminants from the soil (Gibb *et al.*, 1997a). Mechanisms for the detoxification of heavy metals are present in earthworms and have been studied using high-resolution proton NMR. Tissues from individuals that had been exposed to a range of Cu(II) concentrations (40-160 mg kg^{-1} of soil) for 110 days were extracted, and the extracts analysed. This revealed a large number of small metabolites with little variability present between individuals. It was observed that the level of histidine, present at low levels in controls not exposed to copper, increased in response to increasing levels of exposure, whilst levels of other amino acids remained constant. It

was therefore hypothesised that free histidine was accumulating in the metabolic pool of earthworms exposed to toxic levels of copper, and that the increase in concentration resulted from either disruption of the excretion of this amino acid or its synthesis as part of a detoxification process. The level of histidine may therefore act as a biomarker for copper exposure.

Similar studies on earthworms have investigated the molecular toxicology of halo-aromatic compounds. Before this work the only data available were estimates of median lethal concentrations. None of the earlier studies had investigated the biochemical effects of the halo-aromatic compounds, and no biomarker information was available. A study using NMR spectroscopy and PCA methods similar to those described above was undertaken. In this study a problem that is commonly encountered in this sort of work provided a challenge. This was that there was variation due to 'noise' from the spectrum. The problem was overcome by calculating the mean 'noise' level of an area of the spectrum that contained no detectable metabolite peaks. The value was then subtracted from the rest of the spectrum before the application of multivariate pattern recognition. This led to the identification of over 30 endogenous metabolites in the species (Warne *et al*., 2000). Increases were detected in levels of several amino acids, along with changes in the levels of citrate and succinate. The most significant change was in the level of alanine, measured using the peaks associated with the β-methyl group. A combination of the changes was proposed to provide biomarkers of halo-aromatic toxicity in this annelid. Since NMR analysis yields information on the range of important metabolites in one run, and since acquisition times for each sample

are short the investigators suggested that this type of technology had potential for the use in ecotoxicological screening.

Other earthworm studies have highlighted some disadvantages of using whole body homogenates in metabolomic studies, where the concentration or depletion of biochemicals within a specific tissue or fluid could be obscured by residual macromolecules such as proteins and lipids (Gibb *et al*., 1997b). There are also extra sample preparation steps to be considered compared to biofluid analyses, and these may lead to differences in the extraction efficiency for different groups of analytes, and hence introduce artefacts. This prompted an investigation into the biochemical effects of the compound 3-fluoro-4-nitrophenol (3F4NP) on the earthworm using coelomic fluid rather than tissue extracts. Since removing a sample of coelomic fluid is a non-destructive procedure, the same animals could be compared before and after poisoning. The composition of the earthworm coelomic fluid was characterised using one- and two-dimensional proton NMR spectroscopy with the compounds observed recorded along with their respective chemical shift (Bundy *et al*., 2001). The spectra following treatment were then compared with the baseline (control) spectra obtained before dosing. This study revealed that levels of acetate and malonate were lowered and that this was accompanied by an increase in succinate levels. This pattern of response could be used as a biomarker of toxicity following exposure to 3F4NP. It also provided a demonstration of the suitability of coelomic fluid as a suitable substrate for NMR measurements in toxicity testing. There was only one

disadvantage: broad peaks (thought to be from proteins) were present in the spectra, even after centrifugation of the sample.

Some other terrestrial invertebrates have been used in metabolomic studies of toxicity. Most of the studies in this area have characterised the low molecular weight components found in tissue extracts of whole organisms. Two terrestrial isopods, *Onicus asellus* and *Porcellio scaber*, the diplopodous arthropod, *Glomeris marginata* and a pulmonate gastropod, *Arion subfuscus* have been studied in addition to the two earthworm species *Lumbricus rubellus* and *Eisenia andrei* studied (Gibb *et al*., 1997b). Differences were detected between the metabolic profiles of the various species, both in terms of actual metabolites detected and in the ratios of abundances in metabolites that were present in all species. Many endogenous metabolites were assigned and these are available to support future studies in invertebrate species. These organisms have potential to be used as indicators of pollution in ecotoxicological screening. In order to make this feasible it would be necessary to develop high throughput NMR pattern recognition techniques, with the use of automatic data reduction similar to those described above for use in drug development and clinical studies.

A further area of study on terrestrial species is that of the mammals where there is extensive use of metabolomic data in the study of rat and mouse models in conjunction with clinical studies focussing on liver and kidney toxicology of pharmaceuticals (Nicholls et al., 2001, Wei et al., 2009, van Ginneken et al., 2007). Where blood, serum, urine and tissue extracts have

been analysed along side histopathology of these organs to characterise the toxicity pathways and are more closely linked with the clinical studies described above. The number of mammal studies of an environmental nature is rather more limited. A study has been conducted on the comparative biochemistry of three wild animals compared to the laboratory rat (Griffin et al., 2000). Proton NMR spectroscopy was used to investigate urine and blood plasma composition and high resolution magic angle spinning (HRMAS) of intact kidney samples of the bank vole (*Clethrionomys glareolus*), wood mouse (*Apodemus sylvaticus*), white toothed shrew (*Crocidura suaveolens*) and the Sprague Dawley rat strain for the study of inter-species metabolism. The group found that the species were separated by their urinary profiles, while study of the tissues identified differences in lipid concentration and composition, with the wild species exhibiting mush higher concentrations of triglycerides. The study demonstrated that adaptation to natural diet affects renal and urinary profiles leading to the conclusion that comparisons between laboratory animals and wild species may underestimate the impact of lipophilic xenobiotics.

1.4.3. Environmental studies (aquatic)

Although there have been a number of metabolomic studies of toxicity in terrestrial invertebrate species as described above, there have been fewer investigations in aquatic invertebrate species, though the number has increased in recent years.

There has been a comprehensive study of the effects of sub-lethal stress on the abalone, *Haliotis rufescens* with the majority of work completed *in vivo* using the whole organism. A chemical stressor was introduced through custom designed flow through system using ^{31}P NMR to study changes in cellular respiration (Viant et al., 2002, Viant et al., 2001). The advantage of using the whole organism in a flow through system is that the change in cellular energetics can be observed through time as the insult is delivered. The use of ^{31}P NMR is ideal for observing changes in concentrations of metabolites involved in energy metabolism since the key compounds are phosphates.

Another study, using proton NMR metabolomics, on *Haliotis rufescens* was conducted to investigate withering syndrome (Rosenblum et al., 2005). Samples of both foot and digestive gland from healthy, stunted and diseased abalone were used to analyse changes in metabolic profiles of the individuals. PCA was used to distinguish between the profiles of the three groups of animals and it was also possible to identify biomarkers associated with the disease. The metabolomic technique also provided mechanistic information on the effect of the disease. Clinical studies suggested that withering syndrome involves starvation, and this is characterised by the degradation of lipids, glycogen and amino acids. Marine molluscs are thought to display a decreased reliance on fatty acids compared with mammals and freshwater molluscs, and to use amino acids instead. The results of this study are consistent with this theory since a significant decrease in the concentrations of many amino acids was observed in diseased individuals, and a similar pattern

was observed in stunted individuals following 46 days of starvation. This indicates similarity between biochemical pathways of both diseased and stunted animals.

There have also been metabolomic studies into aquatic vertebrate species, investigating both environmental and chemical stress in fish. Several studies have investigated the effects of toxic stress on the metabolism of developing embryos (Viant *et al*., 2005). These methods are able to extend the traditional risk assessment tests for chemicals that are based upon non-specific end points such as mortality and hatching success. The Japanese medaka (*Oryzias latipes*), an established model organism in developmental toxicology, has been utilised to investigate the toxic actions of several chemical stressors. Another study investigated the toxic actions of trichloroethylene, a common ground water contaminant, known to produce pathological abnormalities in rats (Saillenfait *et al*., 1995). This group were able to show that all exposure concentrations (8.76, 21.9, 43.8, 87.6, 175.0 mg L^{-1}) of this chemical exhibited a detectable metabolic perturbation. Using hatching success as an indicator the no-observable-adverse-effect-level (NOAEL) concentration of trichloroethylene was estimated to be 164.0 mg L^{-1}. Twelve metabolites in the NMR profile showed a dose-response relationship, with the largest change being that of histidine (1.72-fold increase). Although a relatively small increase in metabolite concentrations were observed they were found to be significant and the potential of using metabolomic analysis as an unbiased screening assay was demonstrated.

A further study using the same model species investigated the toxic action of the herbicide dinoseb (Viant *et al*., 2006b). After application to crops, this chemical has been found to contaminate drainage waters and is highly toxic to fish (96 h LC_{50} 58 ppb in channel catfish) and other non-target organisms. This group used two methods of investigation. Firstly non-invasive *in vivo* ^{13}P NMR as this method allows the detection of the energy status of the live animal in real time by measuring ATP and phosphocreatine. The embryos were examined in a recirculating system that delivers oxygenated water to a NMR tube. The second method used ^{1}H NMR metabolomics to investigate other metabolic perturbations that correlated to the changes in energy status. It was found that the phosphometabolites changed significantly after 110 h exposure to 50 and 75 ppb concentrations of the herbicide. There was also an increase in lactate whilst ATP, alanine and tyrosine decreased. The authors hypothesised that the decrease in amino acids could be due to their utilisation in increased energy production, and were characteristic of sub-lethal metabolic depression of energetic resources.

Another study investigated the action of three pesticides (dinoseb, diazinon and esfenvalerate) in the early developmental stages of Chinook salmon (*Oncorhynchus tshawytscha*). The investigation was initiated as a result of the decline in the health of fish in the spawning grounds in the Sacramento River, California (Viant *et al*., 2006a). Pulses of these pesticides in the river waters have been shown to last for several weeks and these often coincided with periods when the salmon were spawning. It was suggested these pulses of pollutant may have a detrimental effect on the eggs and alevins. The three

pesticides were chosen as they have a high toxicity to aquatic life with differing modes of action. A ^{1}H NMR metabolomic study of the metabolic pool of eyed eggs and alevin extracts was conducted and was coupled with HPLC-UV measurements of phosphorylated nucleotides and phosphocreatine. Mortality measurements were taken over a 96 h exposure period. Mortality was reported for the highest concentrations of dinoseb (250 and 750 ppb) and diazinon (100 ppb) with no mortality detected due to esfenvalerate. Interestingly, alevins exposed to all concentrations of esfenvalerate exhibited growth abnormalities, whilst the diazinon exposed eggs hatched and developed normally.

The phosphorylated nucleotides in the alevins varied between the different pesticides and this was related to dose. These metabolic changes disappeared however once the fish were placed in clean water for 14-days. The PCA scores showed a dose-response relationship between pesticide concentration and the sub-lethal effects. The analysis of the alevin extracts showed concentration dependant sub-lethal affects for both diazinon and esfenvalerate exposures. The fish exposed to diazinon were shown to have elevated levels of the aromatic amino acids, phenylalanine and tyrosine as well as isoleucine, leucine and valine, implying the induction of proteolysis. The skewing of the trajectory of the dose-response relationship with exposure to diazinon was interpreted to imply a change in biochemical perturbations. It was suggested that the mortality data supports this theory as at the higher concentration (20 ppb) the trajectory shifts and the mortality increases from 0 to 13 %. Overall the study demonstrated that ^{1}H NMR was more sensitive

than HPLC-UV technique and the metabolomic approach has potential benefits to identify dose-response relationships and the biochemical mechanism underlying the different modes of action of the pesticides.

A further study investigated the long-term response of juvenile salmon (*Salmo salar*) to handling stress. ^{1}H NMR coupled with ultra high performance liquid chromatography-mass spectrometry (UPLC-MS) was used as the analytical tool (Karakach *et al*., 2009). The profiling of fish plasma showed a difference between the controls and after one week of handling. These differences peaked at week two of handling and then there was then a decline over weeks three and four. The changes were associated with the increase in the metabolites, choline, lactate, alanine and valine and a decrease in lipid content, suggesting a pattern of heightened metabolism due to the stress provoked. The fact that these levels then declined in the third and fourth weeks suggested some ability to adapt to the stressful environment.

1.5. Conclusions

The utility of NMR based metabolomic studies in the investigation of the toxicity of xenobiotics in invertebrate species has been demonstrated by the studies described above. The methods developed for medical and environmental applications provide a basis for extending metabolomic studies to a wider range of aquatic species that have been used over the years to investigate the effects of waterborne pollutants. With increasing emphasis on the need for environmental screening using biological end points as well as

chemical end points, work in this area looks increasingly useful. Since traditional toxicity testing is generally based on a single end point such as mortality, limited information is available on the mechanism of toxicity. The ability of metabolomics to provide relatively rapid, multi-biomarker information, and its suitability for incorporation into studies to assess chronic effects of toxicants, could enable it to be incorporated into routine biomonitoring of water quality. As has been demonstrated by the COMET project (Lindon *et al*., 2003), automated sample injection techniques can allow the analysis of up to 200 samples a day. Once a suitable database of assignments of the major metabolites, and profiles from intra-specific studies has been established, then screening could be used for the early stages of diseases, and exposure to toxicants in key aquatic species. NMR can be used as a screening tool for profiling, detecting pertubations to metabolism due to both environmental and chemical stressors as well aiding the identification of potential biomarkers of toxicity (Samulsson and Larsson, 2008).

As the technology is advancing more novel experiments, investigating a wider variety of subjects is taking place and the advent of regulation of the analytical methods used is beginning to emerge. Several recent articles have put forward the idea of 'standard methods' for the reporting of metabolomics analyses (Castle *et al*., 2006, Morrison *et al*., 2007). This has been further enhanced by inter-laboratory studies to evaluate the effectivness of ^{1}H NMR metabolomics between international laboratories. This has arisen for both clinical (Lindon *et al*., 2003) and environmental studies (Viant *et al*., 2009). The idea is to meet the fundamental requirements necessary to be adopted

for clinical and environmental regulation and risk assessments. The main focus of this work is the defining of the minimum requirements for laboratory practices and incorporates, sample collection, sample preparation, metabolite extraction, NMR data collection, data processing and multivariate analysis. By implementing these simple protocols across the different laboratories, the data collected was found to be sufficiently comparable between institutions. PCA was able to discriminate between the sets of samples, with the same biomarkers discovered by all the test laboratories. A draft report of REACH (Registration, Evaluation and Authorisation of Chemicals) put forward by the European Commission (Hengstler *et al*., 2006) has included support for emerging, innovative, techniques including 'metabolomics'. This would suggest that the prospect of metabolomics becoming part of regulatory methods is promising.

The present study was undertaken to investigate the toxicity of pesticides in two invertebrate species that have been used as model organisms in screening. The blue mussel *Mytilus edulis* has been used in the assessment of environmental quality in marine and estuarine locations. This organism has been used in the Mussel Watch programme in the UK and on mainland Europe (Baldwin and Kramer, 1994a, Kramer et al., 1989). The larva of the midge *Chironomus sp*ecies has been used as an indicator of water quality and of the potential toxicity of pollutants in freshwater environment (Buchwalter et al., 2004, Callaghan et al., 2001, Conrad et al., 1999, Crane et al., 2002, Pery et al., 2004, Pery et al., 2003b).

1.6. Aims

- To investigate the toxicity of pesticides on the impact to metabolism of the two invertebrate species, *Mytilus edulis* and *Chironomus riparius* used as model organisms in ecotoxicological screening.
- To develop a simple preparative sample technique to extract the low molecular weight metabolites from the tes species and utilize a 1HNMR metabolomics methodology utilizing to qualify and quantify metabolic perturbations induced by the chemical stressors.
- To identify the metabolic profiles of the model organisms under 'normal' steady state metabolic activity and to induce both environmental and chemical stressors for comparison.
- To utilise robust multivariate statistical analysis methods to identify the metabolic disturbances of the stressors and validate these results.
- To contribute to biomarker discovery used in ecotoxicology screening of water quality.

Chapter 2

Method development

2.1. Introduction

High field ^{1}H NMR spectroscopy was the prime selected methodology to be used in this metabolomic study as it has the advantage of a simple method of sample preparation and is completely unbiased in the metabolites it can identify. Other methods using Gas chromatography/mass spectrometry and Liquid chromatography/mass spectrometry need extensive sample processing and certain conditions need to be met to qualify certain metabolite species with low volatility. Therefore derivatization may be necessary, usually by silylation of hydrogen rich functional groups (Britz-Mc kibbin and Terabe, 2003, Wagner *et al*., 2006, Williams *et al*., 2005, Wilson *et al*., 2005). This method has been used in combination with multivariate chemometric techniques (principle component analysis (PCA), and discriminant analysis (DA)(Holmes *et al*., 1998). This can provide a robust, efficient non-destructive method of producing high volume data sets of metabolite composition of organism tissues and biofluids (Gibb et al., 1997a, Lenz et al., 2003). This chapter aims to bring together the methods of sample preparation, metabolite extraction and spectral analysis (Deprez *et al*., 2002, Le Belle *et al.*, 2002). These are used to qualify and quantify the extractable metabolites from the different sample types. Muscle tissue from the marine mollusc *Mytilus edulis* and pooled whole body (10 larvae) homogenates of *Chironomus* larvae. The developed method was used to analyse extracts of mussel and chironomid tissues. The investigation was to gain an understanding of the biological response of the two species when exposed to chemical and environmental stressors. In order to obtain reproducible measurements of the profiles of

small metabolites in the tissues it was necessary to develop an appropriate extraction protocol. There are many techniques for this purpose described in the literature and these were compared to find the most suitable method of extraction for use in this study (Daykin *et al*., 2002, Le Belle *et al*., 2002). Reliable and efficient extraction and preparation procedures are needed to reduce the variability between the samples due to the various stages in extraction and concentration processes (Lin *et al.*, 2006). It is also desirable to minimise the number of preparation steps before analysis to reduce the variability of extraction. Extraction solvent mixtures (acetonitrile/water, methanol/water, methanol/chloroform/water and perchloric acid) were evaluated on the basis of yield, reproducibility, ease and speed. Lin *et al.*,(2006) concluded that these solvent mixtures provided acceptable extractions, and were easy and rapid to use. In this case the methanol/chloroform/water method was preferred (Lin *et al.*, 2007). The use of acetonitrile/water as extractant gave the highest reproducibility, and had the advantage of low extraction of lipids and macromolecules that can interfere with the NMR analysis (Lin *et al.*, 2006).

2.2. Sample preparation

In the investigation in the mussel the first step of was to decide which tissue would be the most appropriate to use for the extraction of low molecular weight metabolites. It was important to select a tissue that would give a satisfactory extraction of metabolites whilst minimising interference from external sources of contamination. It was important therefore that the tissue

was easy to remove from the rest of the body of the mussel without contamination from the hepatopancreas as there was a possibility that ingested material from this could cross-contaminate the samples. This would add noise (making standardisation unreliable) to the analysis of the test population since metabolites would be added to those extracted from the mussel tissues, and would appear to be part of the metabolic profile of the mussel. This could potentially lead to false conclusions, and would add to the variation between samples. Previous studies have used the foot muscle of *Abolone* another marine mollusc species (Viant et al., 2003) and adductor muscle, mantle and gill tissue from *Mytilus* species have also been used to study the effects of environmental stress (Hines et al., 2007). With these parameters in mind the foot muscle was selected as the tissue of choice in this study. The foot could be removed from the mussel quickly and easily without interference from other tissues. The bivalve shell was opened by cutting the adductor muscles, any byssal thread was removed, and the surface of the animal was washed with a stream of distilled water to remove any contamination that may have been picked up from the environment, and dabbed dry. It was important to remove as much foot tissue as possible from each sample as the size of foot varies between organism and unhealthy mussels could display reduced muscle content (Widdows et al., 2002). The next step in the preparation process was sample preservation as metabolic actions of the tissue samples have to be preserved in their original state at the time of collection. Once removed the samples were place in pre-labelled aluminium foil parcels and were then snap frozen in liquid nitrogen (ca. –70 °C). Samples were held under the surface of the liquid nitrogen until all

bubbling ceased and were stored at this temperature until all samples were ready for the extraction process.

For the chironomid species (*Chironomus riparius*) whole body samples were needed. Therefore the amount of extractable dry weight of tissue was investigated. Due to their small size it was concluded that the fourth instar larva was to be used throughout the study since they would yield greater amounts of material for extraction. Since a single larva would not yield enough material for analysis, pooled collections of five and of ten individuals were investigated. Both provided collectable amounts of wet material, but once lyophilised only the larger ten pooled organisms provided enough material (> 100 mg) for the extraction procedure.

2.3. Metabolite extraction

The extraction method is one of the most important steps of any metabolomic study as this procedure provides the material for the final analysis. The first step was to break down the sample matrix to release the metabolites to facilitate extraction from the cellular material. It is at this particular point in the preparation process where the majority of sample variation and error can be introduced. The grinding of the tissue into a wholly homogeneous state can be extremely difficult, especially when using a pestle and mortar. It was found that by keeping the whole apparatus cooled with liquid nitrogen, an efficient grinding could be achieved. The pestle and spatula were stored in a small Dewar (Fisher Scientific, Loughborough) containing liquid nitrogen to prevent

any heat affecting the tissues as they were ground. The mortar itself was placed in a block of polystyrene to act as an insulator and care was taken to ensure that until the tissue was powdered the sample was always covered with liquid nitrogen. This ensured that the tissue remained frozen and brittle throughout the grinding process and reduced the incidence of sample fragments being lost from the mortar. The tissue was ground until a fine consistent powder was produced without allowing the sample to thaw at any stage. It was important at this point that the tissues were ground to as homogeneous a state as possible to provide a consistent surface area for the extraction stage. Any inconsistency here would manifest itself in the subsequent analysis as variability in metabolite concentrations between samples. Potentially this could increase the background noise of the analysis or even give separation of samples on the basis of the extraction procedure rather than on differences due to treatment of the animals. The samples were then lyophilised in 2.0 mL Eppendorf vials to remove any remaining water from the tissue. The lid of the vial was left lose so that the water can escape. It was important that the lyophiliser was properly cooled before the addition of the samples to reduce the chance of the sample ‘boiling’ under the vacuum conditions and therefore the loss of sample. After at least 24 hours the samples were removed and were stored below -20 ºC until needed. In recent environmental studies the pestle and mortar approach has been replaced by the use of mechanical bead based homogenisers to increase consistency and increase sample extraction volume (Wu et al., 2008).

A known amount (100 mg) of each sample was taken in order to enable comparable estimates of metabolite concentrations in the tissue. The extraction of metabolites from the solid matrix of protein and lipid material that would interfere in the NMR spectroscopy was optimised for the mussel foot tissue. An important consideration was to simplify and to reduce the number of steps within the extraction process, since the greater the number of steps the greater will be the error. A simple solvent extraction procedure using acetonitrile was investigated, since this solvent is effective in precipitating any macromolecules in the solution. A set of seven samples was extracted with various concentrations of acetonitrile and $^{2}H_2O$ to find the ratio that gave the best yield of low molecular metabolites. The set comprised of $^{2}H_2O$ (100%) 70/30 %, 60/40 %, 50/50 %, 40/60 %, 30/70 % $^{2}H_2O$/ACN and ACN (100%). In all cases acetonitrile was added first and left for a few minutes to precipitate any proteinacious material, and then the $^{2}H_2O$ was added to complete the extraction. The extract was frozen in liquid nitrogen, and lyophilised. The resultant ^{1}H NMR spectra were compared. The use of $^{2}H_2O$ on its own gave a very low yield and uneven baseline compared with the solvent extracted samples (Fig. 2.1).

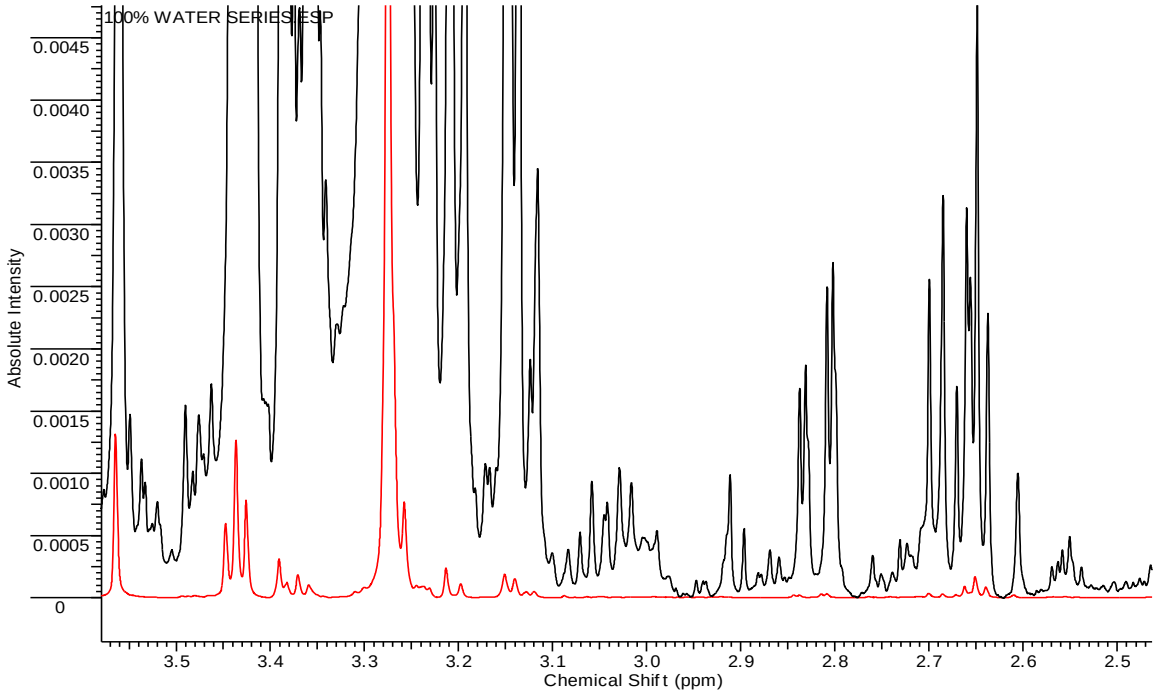


Figure 2.1: Comparison of metabolite yields obtained with water (^2H_2O) extraction with that obtained using 30/70% (^2H_2O/ACN). This shows the significant increase in quantity of extractable metabolites obtained using the solvent extraction mixture.

Large quantities of foam were produced during the extraction and reconstitution processes due to the high lipid content of the tissue. This lipid came not only from the cell contents but was also released from the lipid membranes of the disrupted cells. These lipids and other macromolecules can contribute to problems with shimming and baseline corrections during the NMR analysis due to particulate or aggregates causing local inhomogeneity in the magnetic field. As the proportion of acetonitrile content increased the spectra improved until at the higher concentration (30/70 % ^2H_2O /ACN) the spectral profile started to deteriorate, so the concentration ratio of 40/60 % ^2H_2O /ACN was found to produce the best results and used throughout subsequent experimentation. Where acetonitrile (600 μL) was added, and thoroughly vortex mixed, followed by the addition of ^2H_2O (400 μL), mixing, and then centrifugation in a micro-Eppendorf desk centrifuge (13,000 rpm for

10 min) to remove cell debris and precipitated protein. The supernatant was transferred, without disturbing the pellet, to a pre-washed (^2H_2O) Eppendorf tube (2.0 mL) and frozen in liquid nitrogen and lyophilised (24 h) to remove all of the solvent to leave the extracted material behind. The dried samples were then stored under anhydrous conditions until NMR analysis

The use of 100 % ACN was investigated and produced an entirely different spectrum since only those metabolites soluble in ACN were present (Fig. 2.2), although the spectra themselves were gave better peak resolution (> 0.5 hz in the reference peak) than when water was present (~ 1 Hz).

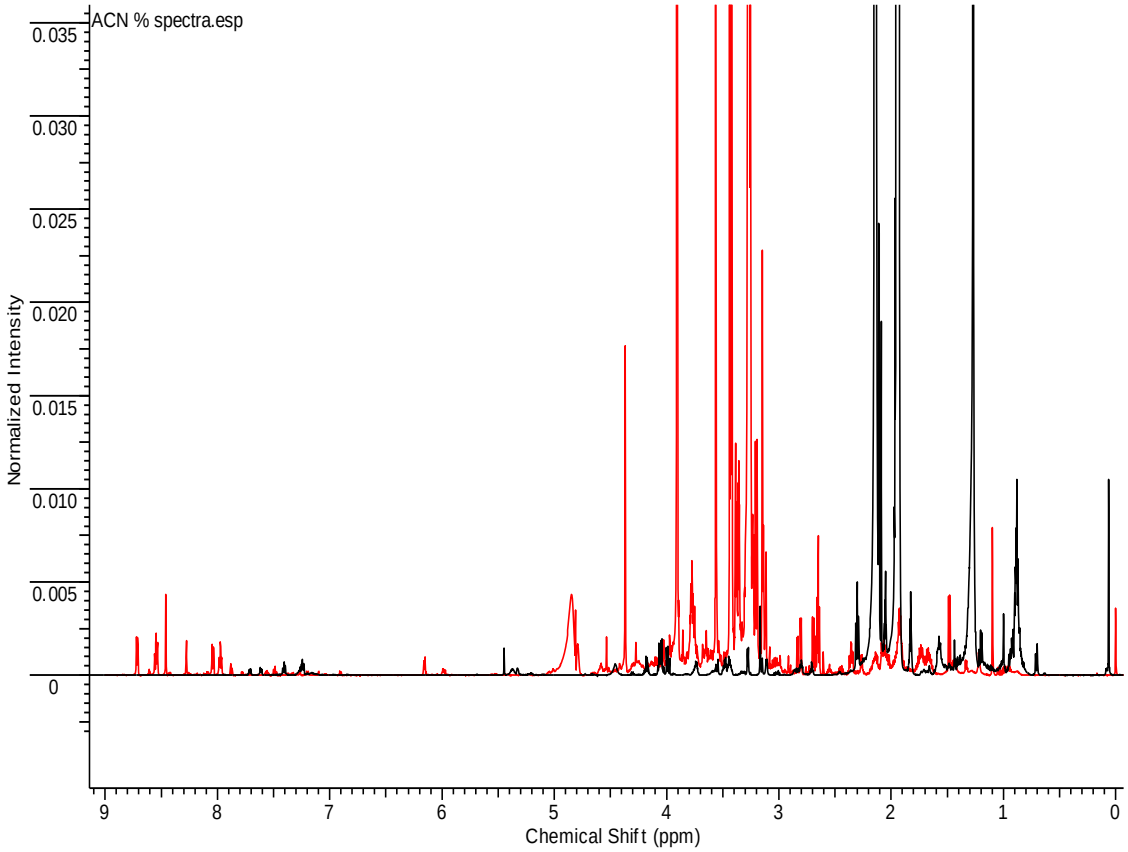


Figure 2.2: The contrasting spectra obtained using a solvent mixture (40/60 % ^2H_2O/ACN) and a single solvent (100 %ACN) for the extraction of mussel foot tissue.

This method could be advantageous providing it yielded reproducible and representative metabolic profiles. The acetonitrile peak contribution was reduced by using deuterated solvent (Sigma Aldrich, 99.9 % (v/v)). The utility of this extraction procedure was assessed by using samples from the lindane exposed mussels (see chapter 3). A preliminary analysis was carried out to see if the control group and the treated group could be separated from each other on the basis of the spectra (Fig. 2.3 and 2.4).

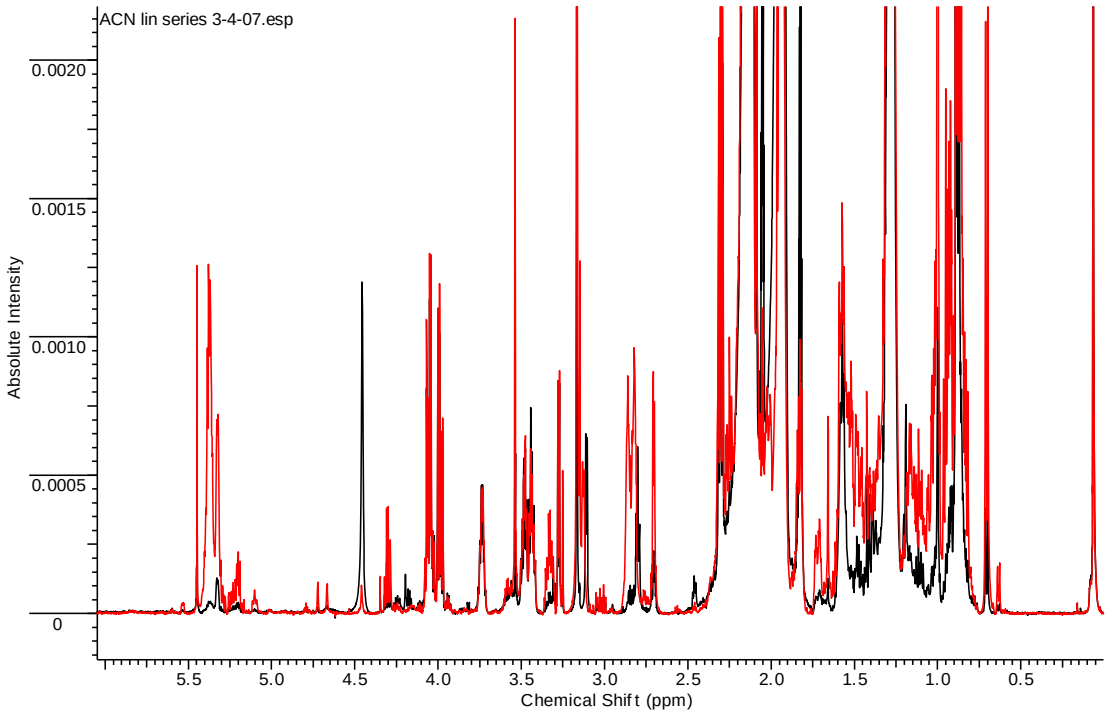


Figure 2.3. Upfield spectra 0.0 to 5.6 ppm of control and lindane exposed (red) metabolite profiles of mussel foot tissue extracts in acetonitrile d^3.

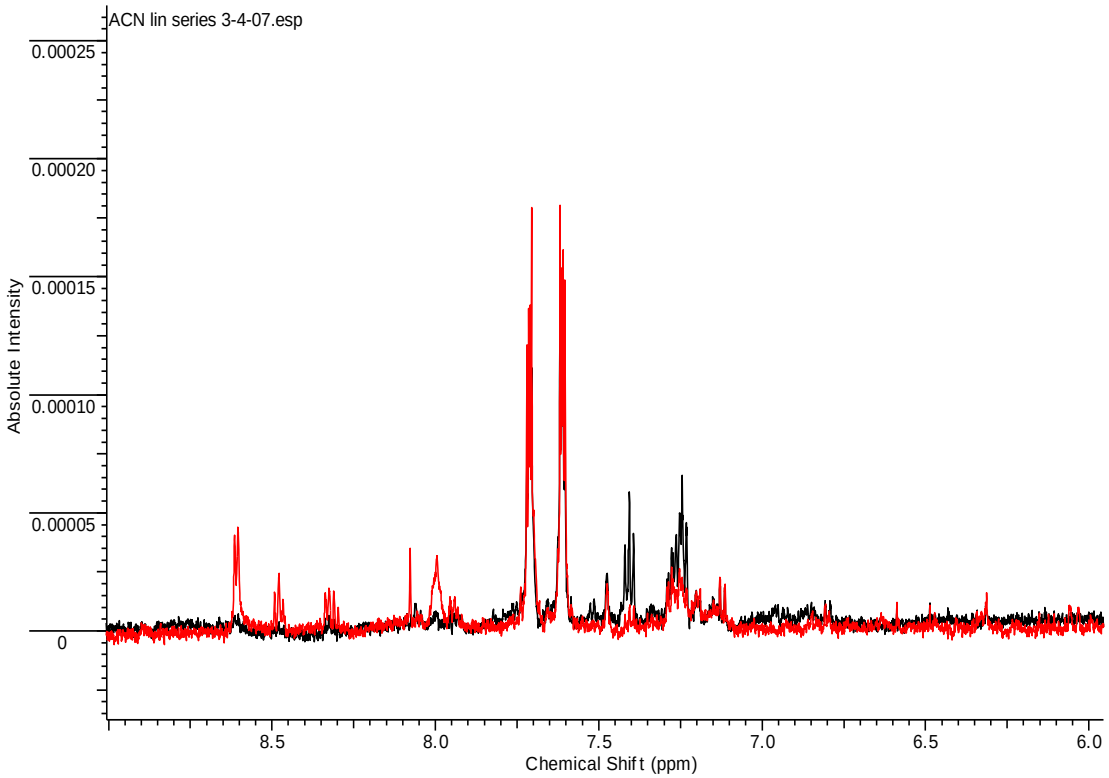


Figure 2.4. Downfield spectra 6.0 to 9.0 ppm of control and lindane exposed (red) metabolite profiles of mussel foot tissue extracts in acetonitrile d^3.

The chironomid study utilised the same solvent extraction process as that used in the mussels experiments and this again gave better spectra than those obtained with water alone. A 100 % ^2H_2O extraction was investigated but this gave an extremely low yield and distorted baseline. The overall metabolite yield obtained from the chironomids was much lower than that obtained from extraction of the mussel foot tissue. This could be due to the extracellular tissue incorporated in the whole body homogenates, such as chitin. This meant that any macromolecule interference would have a proportionately greater impact on the chironomid profiles as the lower metabolite concentrations in those areas affected would be obscured by these broad peaks. Under these circumstances the precipitation obtained with extraction with pure ACN would be advantageous. The interference from macromolecules made data analysis difficult especially for isoleucine (δCH_3,

0.960 ppm and lactate (βCH_3, 1.320 ppm) metabolites that fall directly on top of the broad peaks centred at 0.945 ppm and 1.280 ppm respectively (Fig. 2.5).

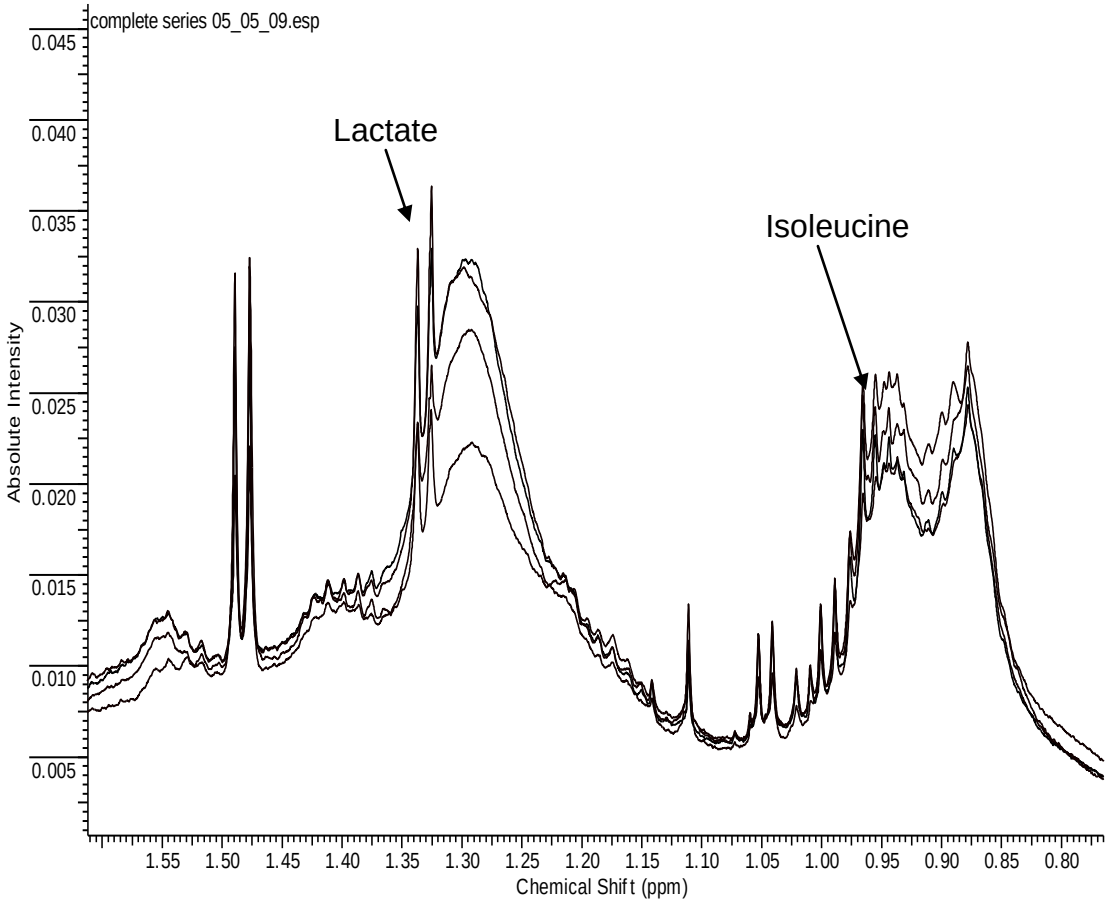


Figure 2.5. Two broad peaks centered at 0.945 and 1.280 ppm correspond to macromolecules in three different extracts of chironomid larvae, that interfere with the integration of the methyl peaks corresponding to lactate and isoleucine centered at 1.320 and 0.960 ppm respectively.

2.4. Controlling sample pH

It was important to be able to control the pH of the reconstituted samples, and this was achieved by the use of a phosphate buffer solution (Sorensen's) to give a pH of 7.0 in all samples. The pH can affect the spectral output of the samples examined by NMR by shifting the peaks of analytes and that of the

concentration of protons (Xiao *et al*., 2009). A Sorensen's buffer solution pH 7.2 was then prepared as follows.

For a 1 M buffer solution the molecular weights of the constituents were used as a reference. The two constituents of the buffer were KH_2PO_4 with a formula weight of 139.09 and Na_2HPO_4 with a formula weight of 141.96. Only a small volume (1 mL) of buffer solution was needed, and the appropriate amounts of the constituents were weighed out, 0.13609g of KH_2PO_4 (A) and 0.14196g of Na_2PO_4 (B). The compounds were needed at different ratios according to the equation 'x mL A + (100-x) mL B' where x = 39.2 at pH of 7.2. Therefore the amount of the two compounds used were A = 0.13609 * 0.392 = 0.05335 g, B = 0.14196 * 0.608 = 0.08631 g dissolved in 1 mL of ^2H_2O giving a pH of 7.2. The buffer was then added to each mussel/chironomus sample (original pH of 6.0) to bring the samples (650 μL) to a pH of 7.0. For this, 50 mM of the buffer solution was required (equivalent to 32.5 μL of the buffer solution per sample).

2.5. GC/MS analysis

2.5.1. GC/MS analysis of possible sample preparation contamination

Although of a lower sensitivity to mass spectrometry any contaminants throughout the extraction procedure and during the analysis such as plasticisers and solvent residues may still be detected if found in high concentrations. These contaminants need to be identified and quantified in

order to assess whether the tissue extracts prepared for NMR analysis have picked significant contamination during the extraction procedure. Therefore two procedural blanks were prepared using following the same sample preparation and metabolite extraction methods (sections 2.2 and 2.3), but with no tissue present. The blanks were then analysed by GC/MS, where a general scan was used to determine the identity and quantity of any contaminants. The resultant samples were extracted with 1 mL of *n*-hexane ready for injection. The initial oven temperature was set to 60 °C with the back inlet in splitless mode and an oven temperature of 250 °C. The pressure was set at 19.49 psi and a purge flow of 100.0 mL min^{-1} for 2 mins giving a total flow of 106.1 mL min^{-1} of helium. A capillary column, (Factor Four, VF-Xms, 30m, 0.25 mm, 0.25 μm. Part number: CP8806, Varian) was set to a maximum temperature of 260 °C with a constant flow. This was set initially at 1.3 mL min^{-1} at 19.49 psi with an average velocity of 2 cm s^{-1} and a vacuum outlet pressure. An automatic injector was used with 4 sample and 6 pump washes and an injection volume of 1.0 μL. The syringe size was 10 μL with 8 post injection washes of solvent A (acetone) and 8 of solvent B (*n*-hexane). The final run time for each sample was 38.50 mins.

The resultant spectra revealed a low level abundance (< 500 ppb) of several phthalates and phenols. The source of these was probably plasticizers used in the production process of the glass vials and plastic equipment used in sample preparation. Their presence was unavoidable, and at the low levels detected they would not have a major effect on the NMR spectra obtained for the tissue samples.

2.5.2. GC/MS analysis of test water pesticide concentration

The chemical exposure of the mussel samples was performed using a 24 h renewal static system (sections 3.2.4). Under these conditions, the concentration of pesticide is not constant but declines over the 24 h between renewals. In order to measure the exposure of the animals to pesticides, samples of the incubation medium were taken at 0, 1, 2, 4, 8, 12, 24 h intervals for analysis by GC/MS. Calibration standards were produced from stock solutions containing 1 mg of lindane and a second with 1 mg of atrazine, both in 100 mL of acetone. These were stored in tinted glass bottles. They were mixed thoroughly for 30 min using a magnetic stirrer (AGE, Fisher Scientific, Loughborough) to ensure that the compounds were in a homogeneous solution. From these two stock solutions a set of serial dilutions were produced as a set of working standards for the calibration of the measurement of the nominal concentration. With a starting concentration of 10,000 ng mL^{-1} a set of dilutions using equivalent aliquots in μL were prepared. Where 500 μL of the 10,000 ng stock solution added to 500 μL of acetone gave 5 000 ng mL^{-1}, 200 μL stock added to 800 μL acetone gave 2 000 ng mL^{-1}, 100 μl stock to 900 μL acetone, 50 μL stock to 950 μL acetone and finally 10 μL stock to 990 μL acetone. These gave a final set of standards of 10,000, 5,000, 2,000, 1,000, 500 and 100 ng mL^{-1}, their abundance was then recorded using the GC/MS method described above. Calibration curves for both toxicants are presented in Figs. 2.6. (lindane) and 2.7. (atrazine). These calibrations were used in the analysis of the samples of incubation

media taken during the exposure of the animals to the pesticides (section 3.2.4).

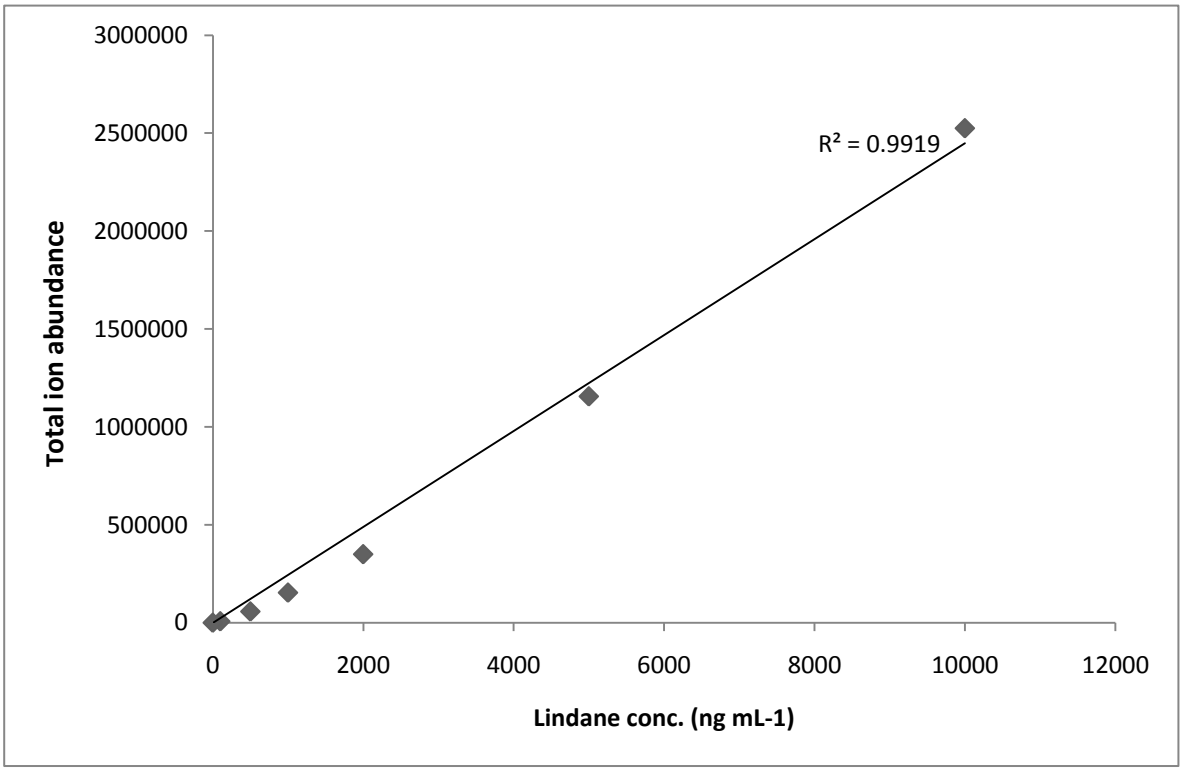


Figure 2.6. GC/MS calibration curve for lindane.

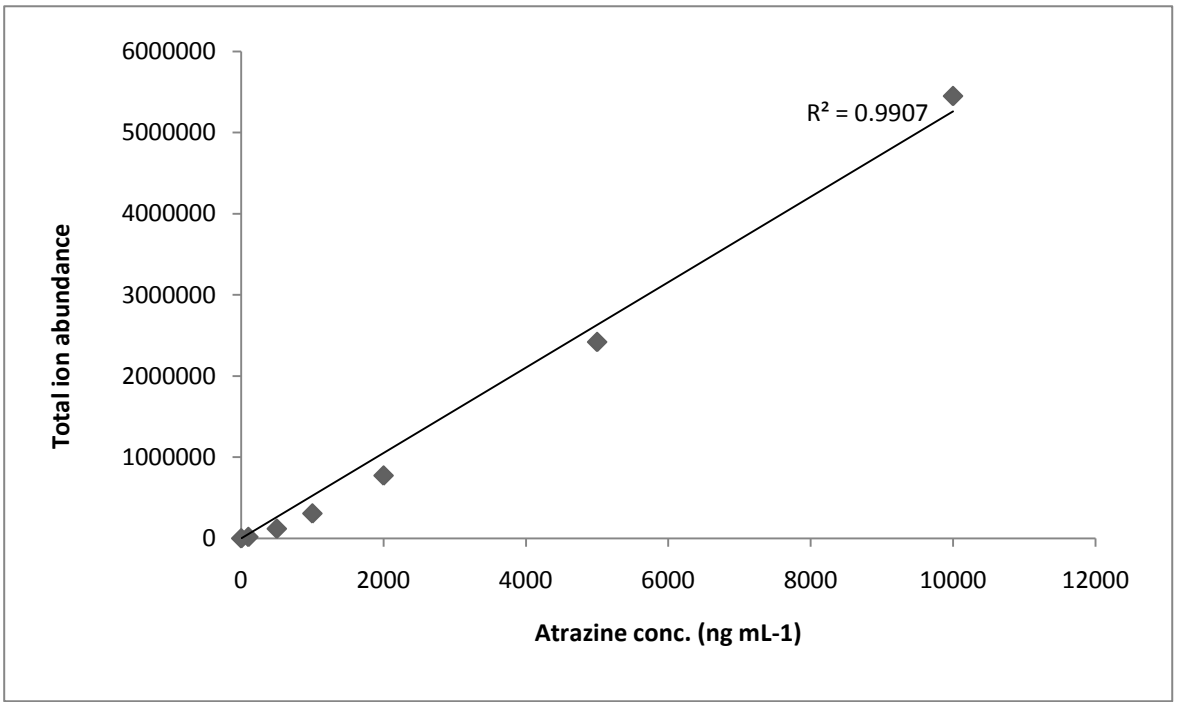


Figure 2.7. GC/MS calibration curve for atrazine.

2.6. NMR Spectral acquisition methods

2.6.1. Introduction

To analyse samples using NMR there are some vital preparatory steps that need to be completed and these follow this introduction. The samples themselves need to have as much water removed as possible and deuterated solvents added as a prerequisite to allow the metabolites within the sample to be visible as all the protons within the water of these biological samples will be detected. By removal and replacement of these with a deuterated solvent (eg deuterated water) the solvent peak will be significantly reduced to a manageable level (Deprez et al., 2002). An internal standard is also essential so that all subsequent samples can be calibrated to this standard on the ppm scale of the spectra. Following on from this the NMR spectrometer also needs to be tuned to sample frequency and optimised focussing on the solvent frequency. Once tuned the spectrometers magnetic field needs to optimise the homogeneity of the magnetic field to give the best resolution for each sample. It is also important to optimise the amount of data collected by the probe is investigated to give the best signal to noise ratio over the time each sample is analysed and a compromise found as yield increases with time but a workable per sample time is needed (Gunther, 1998). One final preparatory step is that of solvent suppression, as although the majority of the solvent (water in this case) has been removed and replaced with a deuterated form, any residual water may still give a large peak of interference around its origin. Therefore a pulse sequence is used that introduces a saturation effect at the frequency of the solvent (water) to effectively cancel out this interference

(Chen et al., 2004). These preparatory steps are covered within this section for the samples investigated giving detailed information on the set up of the spectrometer.

2.6.2. Sample preparation for ^{1}H NMR spectroscopy

The dried extracts were reconstituted in $^{2}H_2O$ (700 μL) and Sorensen's phosphate buffer (1 M, KH_2PO_4/Na_2HPO_4 prepared using $^{2}H_2O$, pH 7.2) (35.5 μL)) and vortexed (30 s). The solution was centrifuged (13,000 rpm) briefly to remove any remaining particulate matter and the supernatant transferred to a 5 mm diameter NMR tube. Sodium 3-(trimethylsilyl)-2,2,3,3-d_4-propionate (TMSP) (3 μL, 0.25 mM) was added as an internal reference standard to which the chemical shift axis can be calibrated (0.00 ppm) and the instrument was locked to the $^{2}H_2O$ frequency.

All analyses were performed on a Unity Inova 600 NMR spectrometer (Varian Ltd, Oxford, UK) equipped with a cryoprobe (held at 25 K) and operating at a spectral frequency of 599.80 MHz at 298 K. One-dimensional (1-D) ^{1}H NMR spectra were obtained using a pre-solvent saturation pulse sequence with a saturation delay of 1.5 s, followed by a 90° pulse (7 μs) and then an acquisition time of 7.5 s. All spectra were recorded with a spectral width of 13.34 ppm, post 16 steady state pulses succeeded by 256 transients that were collected into 60,000 data points over a period of 15 min. In order to improve the base line flatness and thereby integration of peaks a delay of 30 μs was set between the end of the 90° hard pulse and the start of the acquisition period. The resultant data-sets were zero-filled to 131,072 points

and then a Fourier transformation was applied with a weighted exponential line broadening factor of 0.5 Hz. The spectra were manually phased and base line corrected before being calibrated (TMSP at 0.00 ppm) using ACD/Specmanager (ACD/Labs version 9.0, Toronto, Canada). Peaks were assigned by reference to known chemical shifts (Wishart, 2007) using the ACD/Specmanager software. Some shifts were confirmed by spiking samples with standard amino acids (5 μg of each) and comparing the original and spiked spectra (section 2.9).

2.6.3. Lock and shimming

To create a homogeneous magnetic field around the sample the NMR probe needs 'shimming' these are subtle changes in current of the probe coils to create various gradients of desired strength. To obtain a satisfactory spectrum, gradient variations caused by changing the sample, temperature etc. must be shimmed out. Once a sample has been added to the machine the probe head needs to be tuned to the frequency of interest. This is dependant on the solvent used, in this case $^{2}H_2O$. The probe is tuned manually on the probe mount itself, this is achieved by turning the tuner until the wobble meter displays and holds at zero. The probe can then be locked to the ^{2}H frequency, this is a stabilisation process, ensuring the lock power is not as great as the probe signal overloads. Once in lock mode the probe can be shimmed to the particular sample to give the best peak resolution (down to 0.3 Hz for protons). The probe is shimmed across various gradients (z0, z1,

z2, z3, z4, z5, z6, x1, y1, etc). These are known as orders (zero order, first order, second order, etc) and have to be shimmed accordingly, starting at the low order and working up.

2.6.4. Determination of the 90 ° pulse

To further calibrate the radiofrequency field the 90 ° pulse angle rotation needs to be determined. This was achieved by setting the transmitter power (tpwr) to 63. An array process was then set up of the pulse width in increments of 0.1 s starting at 25 s to 32 s. This process is allowed to run and the output examined to determine where the 360 ° pulse is equal to 0. The time at 360 ° can then be used to calculate the maximum 90 ° pulse time as each 90 ° increment will be the same. This was then set as the pulse width of the pre-saturation pulse sequence.

2.6.5. Acquisition time

The acquisition time (at) of the pulse sequence was investigated to find the optimum delay between pulses to give the best data collection within a reasonable time period. Fig. 2.8 shows a single peak series using the range of at; 0.3, 0.5, 1.0, 1.5, 2.0, 3.0, 5.0, 7.5, 10, 15 and 20 s. The longer the acquisition time the greater the amount of data recorded, but this adds to the overall time per sample. An acquisition time of 7.5 s gave the best balance

between quality of data collected and a workable overall acquisition time of 16 min per sample.

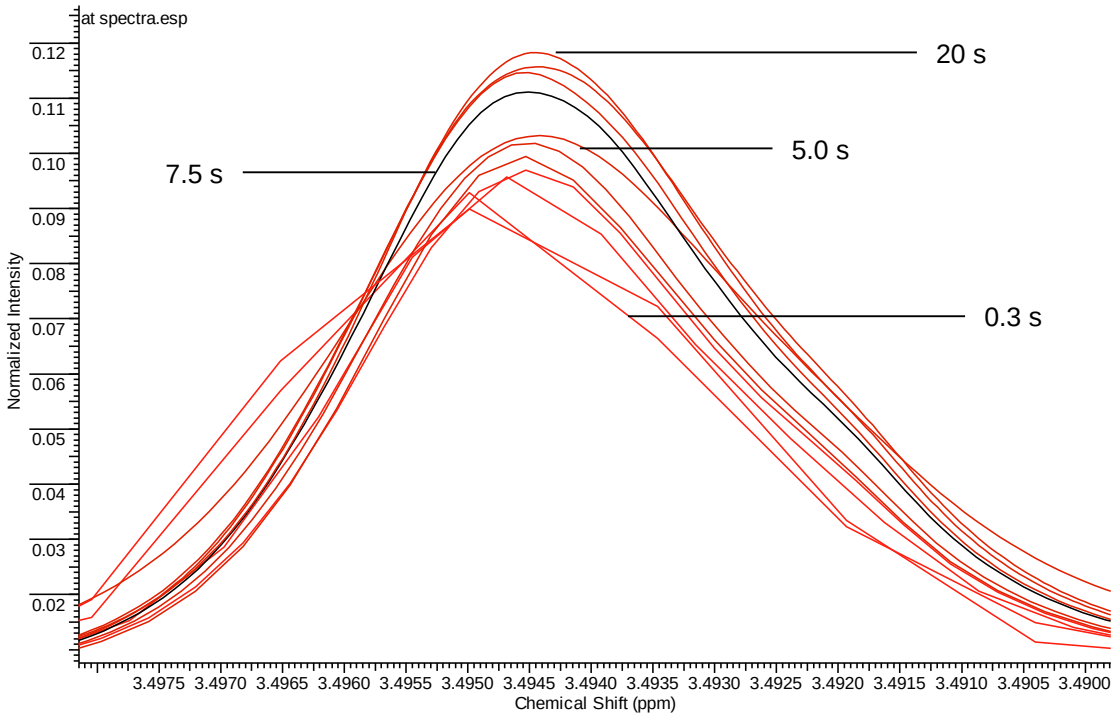


Figure 2.8. A spectral series for a range of acquisition times, the increase in peak size and detail indicates a greater amount of data acquisition.

Below 3.0 s the line shape of the peak was ill-defined this could have been corrected by increasing the zero filling option, but the spectra was still poor. There was a relatively large increase in signal between 5.0 and 7.5 s indicating the need for this length of time delay to record the data. The increase to 20 s gave a small increase in yield, but required a long overall acquisition time (over an hour per sample). As all NMR experiments were completed manually needing time for shimming and adding the sample to the NMR machine an, at of 7.5 s was used to give a consistent set of sample spectra, with a full sample turnover of ~30 min.

2.6.6. Water suppression

A water peak was present in all spectra, and it was necessary to reduce this as much as possible to give a clear spectrum without interference. A pre-saturation pulse sequence (Fig. 2.9) was employed to achieve the required suppression. This type of sequence allows the user to saturate the proton signal at the solvent frequency (water) for a set period of time (1.5 s) and set a minimal power level to avoid baseline distortion prior to the excitation pulse. At this point the instrument was rechecked for good line shape of the TMS peak. A series of experiments was undertaken where an array of values, (satfrq, satpwr and satdly) were introduced to investigated the best set up for saturation of the water peak. It was possible to reduce the water peak to a minimum with little interference in the resultant spectra by an array of the solvent peak and changing the power of the suppression pulse. The addition of a 1 s delay prior to pre-saturation and a saturation period of 1.5 s and an acquisition time of 7.5 s increases the cycle time between excitation pulses to 10 s, thereby giving a true integration value for all peaks recorded.

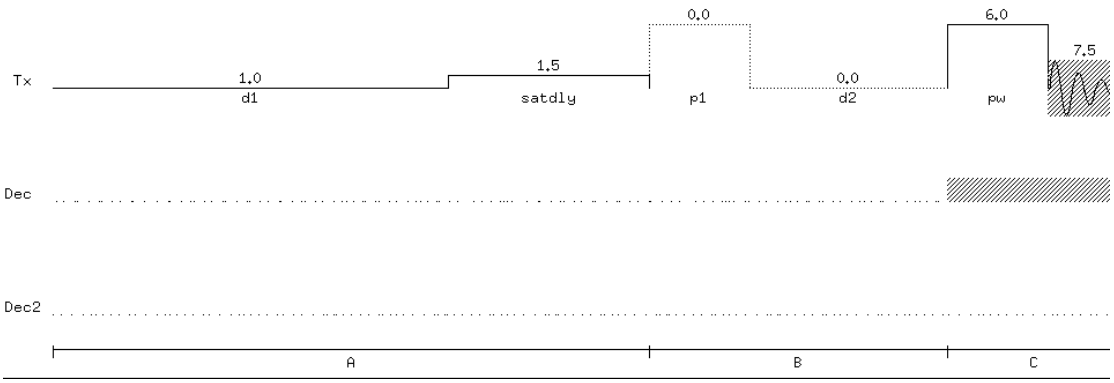


Figure 2.9. Water pre-saturation pulse sequence, d1 is a time delay, p1 is a second pulse (not active), d2 a second delay (not active), pw is the pulse and the shading indicates decoupling (Dec) pulses (not active).

2.7. Binning process in the ACD/Labs software

The fid (free induction decay) was imported to the software and zero filled to 130,000 data points, a 0.5 Hz exponential line broadening function was applied followed by Fourier transformation. Once manually phase corrected, a multipoint polynomial model (order 4) automatic baseline correction was applied across the whole spectrum. This procedure is repeated for all spectra to be analysed and the binning process applied. Once all of the spectra are ready each one had to be imported into a tiled window. At this point the reference peak at around 0.00 ppm was checked throughout the series by using the alignment short cut to see if all peaks are set to 0.00 ppm. If any variation was found the peak was highlighted so that the ppm value was calculated and this then recalibrated to 0.00 ppm using the organiser section. Once this was complete the spectra windows needed to be highlighted in the order you wish them to appear in the series table. A series could then be created where all the spectra were incorporated into one window. The group treatment shortcut was then used so that all the spectra in the series are

treated the same. The integration section was then entered and in options the bucket integration was set up. With either the number of bins or in this case the width of each bin was entered (0.01 ppm) and the intelligent bucketing algorithm set to 50 %. This will allowed the bin size to shift if a peak was cut by a particular bin using the slope of the integral as a guide. The reference was set to the whole spectrum and the operation initiated. Once complete a scan of the bins was taken and any problems with the bucket positions were sorted manually.

2.7.1. Bin exclusion

Each NMR spectrum was reduced to between 0.80 and 9.00 ppm, as areas outside of this contained no relevant NMR data. Any pH-sensitive resonances were compressed into single bins to remove this variability using the 'looseness of fit' operation within the ACD/Specmanager software. The data was trhen exported to an Excel© spreadsheet and bins at 4.70-4.90 ppm (water), 1.91 ppm (residual acetonitrile) and 3.36 ppm (buffer contaminant) were removed from all spectra before analysis. The resultant non-square matrix was analysed using routines in the Minitab (version 14) and GenStat (version 10.1) software packages.

2.8. Statistical analysis

The samples by bins matrix was transposed to give the samples in rows and variables in columns. The principal components analysis (PCA) routine in

GenStat was used to reduce the original standardised data to a number of principal components (PC's). A linear discriminant analysis (LDA) was used to identify those PC's that were important in separating the treatment groups (section 3.3.7). The loadings of the original variables on those PC's were used to identify those bins that contained information that was correlated (loading score > 0.05) with those components. Bins that did not load on to those components were removed, and the chemical shifts corresponding to the remaining bins were investigated to assign them, where possible, to specific metabolites. Standard normal probability plots (Minitab) were used to check for normality of the distributions of each metabolite, and where necessary Box-Cox transformations were used to identify appropriate normalising transformations. This was followed by analysis (one-way ANOVA) of the transformed variables to identify those exhibiting a statistically significant effect of the treatments. A LDA (GenStat) was used to optimise the separation of the treatment groups on the basis of the sets of metabolites (transformed variables). This supervised classification was validated for quality by (mis)classification error rates by conducting permutation tests. The classification labels of the variables in the 'Y' matrix for each dataset were randomised using random number generator. Each dataset had their class labels randomised fifty times, each of the fifty sets were then subjected to further LDA (Genstat) and the classification of the groups were recorded for each new dataset. Error rates were then calculated to indicate the probability that the treatment group separations were derived by chance alone. Plots of the first two discriminant functions were used to map the distances between the various group means, and the correlations between the original variables

and the discriminant scores were used to identify those metabolites that were important in the separation of the treatment groups, and to indicate the directions of the changes with treatment.

2.9. Repeatability study

A study was undertaken to determine whether the sample preparation and analytical procedures produced consistent results. The extraction of metabolites and their reconstitution for spectral analysis was investigated by pooling several homogenised mussel foot samples after the freeze drying step. The combined samples were ground in a pestle and mortar under liquid nitrogen to ensure thorough mixing. This pooled material was then divided into five samples of 100 mg dry weight, and these were reconstituted, using the same method as used throughout the mussel trial (sections 2.2 and 2.3), for analysis. Extra care was taken to ensure that the shimming and phase corrections were as accurate as possible between samples so that the majority of error would be due to the extraction process rather than to steps in spectral analysis. The free induction decays (fid) were processed using ACD/Labs software with the data points count doubled and a 0.5 exponential line broadening function applied before Fourier transformation. All of the spectra were phase and baseline corrected as mentioned before (see 2.7). The spectra were then inspected for any significant variation between the replicate samples. The spectra were consistent except for the larger peaks of betaine, and for variation in the broad peaks around 0.80-1.00 ppm (Fig.

2.10.) and 1.20-1.35 ppm caused by macromolecules. This would suggest that differences in the degree of precipitation of macromolecules between samples were the main source of variation rather than loss of yield of the metabolites. This indicated that care was necessary when analysing peaks in those regions since their areas may be influenced by the macromolecule content. Unfortunately due to time constraints this same procedure could not be repeated for the chironomid study data.

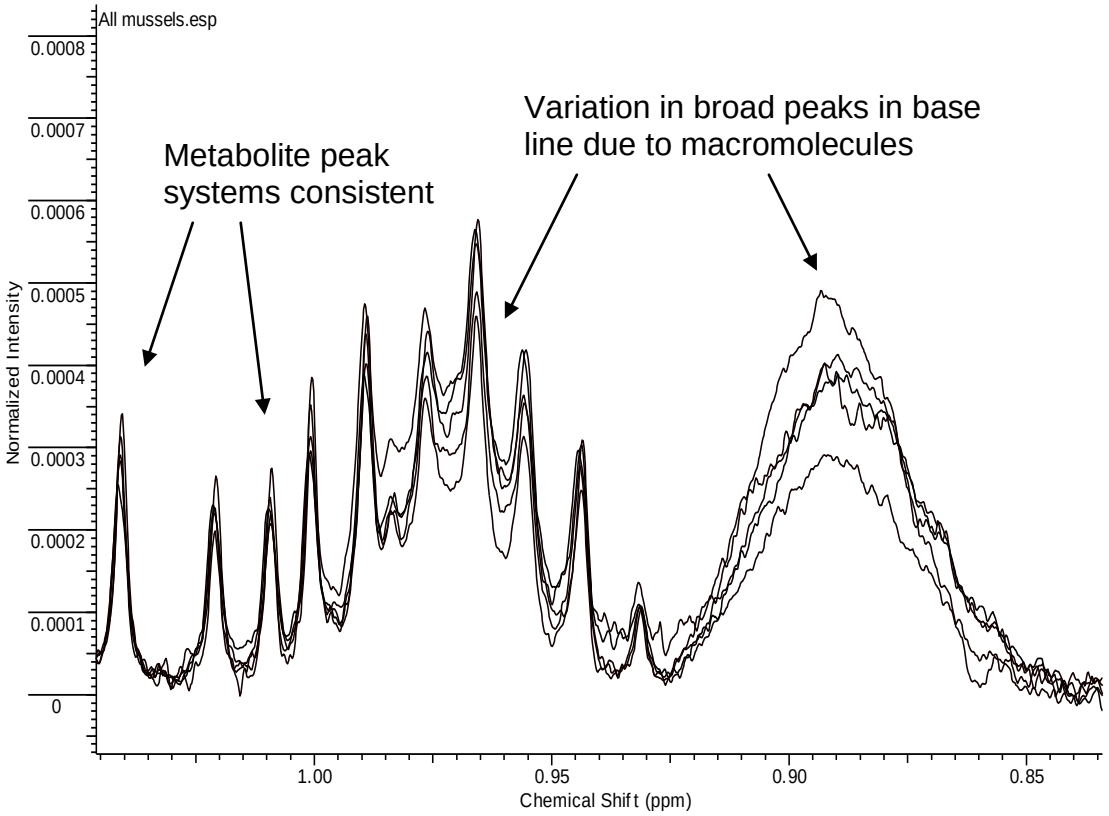


Figure 2.10. *M. edulis* spectrum series showing variation due to macromolecules whilst signals from metabolites are relatively consistent in that the intensities vary only slightly.

2.10. Amino acid fortification experiments in model species samples

The ^{1}H NMR spectrums of the model species show a mass of overlapping peak data shown here in the mussel *Mytilus edulis* (Fig. 3.6). The many different metabolites found within the extractable metabolic pool of these organisms' are represented by one or more of the visible peaks in the spectrum. In order to be able to interpret the spectrum in terms of the various metabolites, it is necessary to assign the various peaks to their respective metabolite. In the first instance each spectrum was examined in great detail and provisional identifications of certain metabolites were made using the chemical shift (ppm) and peak shape recorded in previous studies (Fan, 1996, Hines *et al.*, 2007, Wishart, 2007).

Where possible the use of conventional two-dimensional spectroscopy was used to confirm the amino acid metabolites present in the model invertebrate species *Chironomus riparius* metabolome (see section 4.2.6). In *Mytilus edulis* this was precluded by the very large dynamic range of the betaine peak structures at 3.27 ppm and 3.91 ppm that obscured the 2-D NMR spectra of the other metabolites that were present in much smaller concentrations. In order to confirm the assignments made on the basis of comparisons with published assignments it was necessary to fortify experimental samples with standard compounds. The solubility of samples of pure standard compounds in the solvent mixture used in the extraction process was checked, and all of the metabolites tested (Table 3.1) were at least partially soluble. A known quantity of each metabolite was added to different invertebrate samples of both model organisms. As the quantities of the metabolites within each

sample were not known a sighting experiment was carried out to find a suitable level of fortification. In the first experiment 1 μg of alanine was added, but this did not have a significant effect on the spectrum at the allocated peak regions for this metabolite in a *Mytilus edulis* sample. Stepwise increments of 1 μg were used until a sufficient increase in peak height was observed at 5 μg. Following this a range of standard compounds were added at least to this level, and the resultant spectra were analysed to identify which peaks increased due to this addition, allowing the identification of particular amino acid metabolites within the sample.

Chapter 3

Investigation of the metabolism of *Mytilus edulis* during exposure to chemical and environmental stressors

3.1. Introduction

Many aquatic invertebrates living in marginal environments such as the coastal fringe or estuaries are often subjected to stress because of changing environmental conditions due to natural factors such as tidal events. This is particularly marked for sessile organisms such as bivalve molluscs that depend on maintaining a flow of water over the gills for both feeding and external respiration. These organisms avoid the harmful effects of exposure to low salinities as found in some regions of estuaries at low-tide by closing their two shells (valves) together. There are indications that valve opening is also reduced when there is a low concentration of food suspended in the water column (Kramer *et al*., 1989, Newell *et al*., 2001). While the valves are closed the molluscs are unable to feed and are subjected to hypoxia. These organisms can also be subjected to hypoxia because of for instance, increases in water temperature that reduce concentrations of dissolved oxygen (De Zwaan and Eertman, 1996). In the natural environment mussels are not usually subjected to a single stressor at a time, and this contrasts with most laboratory-based investigations (Hamer et al., 2008). Any factors that limit feeding will impact on scope for growth and reproductive capacity, and hence the time available for normal activity will have a marked effect on the population size, and time necessary for development. This will ultimately limit the distribution of a species depending on their tolerance (Widdows et al., 2002).

Some anthropogenic pollutants are known to have a marked effect on the behaviour, physiology and biochemistry of bivalve molluscs (Donkin *et al.*, 1997), and because of this, their ability to tolerate a range of environmental conditions, and sessile life style, they have been widely used in biological early warning systems (e.g., the Musselmonitor) (Bayne, 1989) and in biomonitoring (Burton *et al.*, 2005, Hellou and Law, 2003). In the latter, the accumulation of pollutants of concern in caged organisms over a fixed deployment period is measured to provide an estimate of bioavailability of substances of concern. Where organisms are sessile it is possible to use wild specimens to detect exposure to pollutants. This involves the identification of suitable biomarkers (physiological or biochemical changes in response to exposure to toxicants) that can be easily measured. The Mussel Watch programme involved the measurement of a fundamental parameter, scope for growth, as a means of assessing the water quality at test sites (Baldwin and Kramer, 1994). Scope for growth is a measure of the overall energy status of an animal based upon the difference between energy intake (measured by food intake) and energy expenditure (measured from respiration rate). That is the amount of energy that is available for growth and reproduction after the basic needs of metabolism have been met.

Aquatic organisms can be exposed to a wide range of pollutants including heavy metals, household products (e.g., detergents), pesticides and high volume, non-polar industrial chemicals (e.g., polyaromatic hydrocarbons, brominated flame retardants, and fluorinated compounds). The levels of these compounds in water bodies can vary in time depending on factors such as

sporadic discharges, seasonal use, water flow and weather events. Usually organisms are exposed to a complex mixture of toxicants rather than to a single substance and so it is difficult to identify the impact of individual compounds (Altenburger *et al*., 1996). Accumulation of a body burden of non-polar organic compounds can lead to non-specific narcosis (Hermsen *et al*., 1994), even in the absence of toxicants with a specific mode of action. In a regulatory context and for risk assessments, the interest is in the toxicity and impact of single compounds, and metabolomics offers a way of identifying the mode of action that is characteristic of individual pollutants. Similar secondary lesions can be produced by a range of compounds, and in some cases the responses to a toxicant could be similar to those observed in healthy animals subjected to environmental stress. For instance if a pollutant has the effect of reducing ventilatory activity and hence feeding activity, then the animals would be subjected to a combination of starvation and hypoxia.

In a study in which *M. edulis* was subjected to chronic (56 day) exposure to high sub-lethal (0.5 LC_{50} at 60 days) concentrations of lindane (0.93 mg L^{-1}), and atrazine (3.585 mg L^{-1}) both compounds produced a decrease in feeding activity and absorption efficiency, and a marked decrease in scope for growth. However, there were differences between the profiles of responses produced by the two compounds. The oxygen consumption of lindane exposed individuals fell to 10% of control levels while for those exposed to atrazine it was 29% greater than that of controls (El-Shenawy *et al*., 2006). The impacts of exposure to lindane at two doses, one high, sub-lethal, and the other below the no effect level, were compared with control animals. In a second study,

the effects of similar doses of atrazine were compared with control animals, and with animals subjected to either starvation or hypoxia. It was not possible to compare the effects of lindane and atrazine directly since the two studies were conducted at different times of the year due to limited capacity of the holding facility.

3.1.1. Biology of *Mytilus edulis*

The marine mussel of the species *Mytilus edulis* are bivalve molluscs found in dense populations around the coastline of the United Kingdom attached to hard surfaces. They are a benthic species found in the littoral zone of the shoreline. Predominantly a sessile species, they attach themselves to the substrate by means of byssal threads, a proteineous material secreted from glands associated with the foot (fig. 3.1). Within this zone the organism may experience large fluctuations in temperature and salinity and are thus eurythermal/haline and able to adapt and respond to these unstable conditions. The fact that some individuals spend extended periods of time exposed to air and thus potential desiccation, reduced oxygen intake and thermal shock. Adaptations to these environmental stressors are related to the functional adaptation of the body of the organism, which is enclosed within two opposing shells and surrounding mantle tissue, forming the bivalve morphology (fig. 3.1). The shells themselves are held together by an anterior and posterior adductor muscles that allow the opening and closing of the ‘valves’ following the tidal patterns of its environment. When submerged the

valves are able to open to allow the inhalation and exhalation of seawater through the mantle of the body to extract food and oxygen. At times of emergence from the water the valves are closed retaining seawater within the shell valves reducing desiccation effects when exposed to air temperatures (Yonge, 1976).

3.1.1.1. Feeding and digestion

The submerged organism uses muscular action to pass water over the four pairs of gill structures known as demibranchs within the mantle cavity. Each demibranch consists of two lamellae which are formed by rows of ciliated filaments. These cilia move the water through the gill structures removing particulates from the inflowing water column (fig 3.2). This flow of water is regulated by the inhalant and exhalant siphons found at posterior of the shell formation. Particulates that enter the gill chamber are bound to the lamellae by a layer of mucus and are carried via ciliated grooves to the labial palps that regulate the food intake into the mouth. Particulates of non food material or excess food are rejected from the mantle cavity into the exhalant current as pseudo faeces as it has not passed through the stomach. Food passing through the mouth then enters the stomach and onto the intestine, where digestive ducts lead to glands where digestion occurs and nutrients are stored (Bayne et al., 1976c) (fig 3.2).

3.1.1.2. Respiration and circulation

The gills are bifunctional structure and this means that in order to maintain significant food intake, water is passed over the gills at a rate that is inefficient for gaseous exchange. Therefore a large surface area is required to support this reduced gaseous exchange across the gill membrane. Huge volumes of water are passed through the mantle cavity supplying the blood with plentiful amounts of oxygen (Bayne et al., 1976d). The membrane wall is also thin reducing the distance oxygen has to travel to the blood. The circulation of the blood through a vascular system is controlled by a heart in the pericardial cavity (fig 3.2) and is distributed throughout the body through five channels, which supply the mantle and related muscles, the gastro-intestinal arteries, the pericardial artery, hepatic arteries and the terminal arteries. The venous system carries blood from sinuses to the kidneys and onto the gills and finally back to the heart. The haemolymph itself contains no respiratory pigments and has an oxygen-carrying capacity equivalent to seawater (Bayne et al., 1976a).

3.1.1.3. Excretion

The excretory system consists of paired kidneys and pericardial glands, the kidneys form U-shaped tubes from the labial palps to the posterior adductor muscle (fig. 3.2). Although kidneys function as excretory organs, excretory products are also lost across the body wall including the gills. Blood filtration occurs in the pericardium and secretion and re-absorption occurs in the

glandular part of the kidney. The dominant excretory product is that of ammonia with a small but significant loss of urea. Large amounts of amino acids are also lost from bivalves these losses have been shown to increase when the organism is under environmental stress such as starvation (Bayne et al., 1976b).

3.1.1.4. Reproduction

The reproductive cycle of the species occurs over an annual season with the formation and ripening of the gonad. There are four main recognised stages within this cycle; developing, ripe, spawning and spent. The development of the gonad commences in early winter, with gametogenesis occurring over the winter months until in early spring the gonad is morphologically ripe. Further gametogenesis can continue into the summer months with favourable feeding conditions. The spawning event extends over a four to six month period with the majority of organisms reaching a spent condition during early autumn known as the resting phase (Seed, 1976).

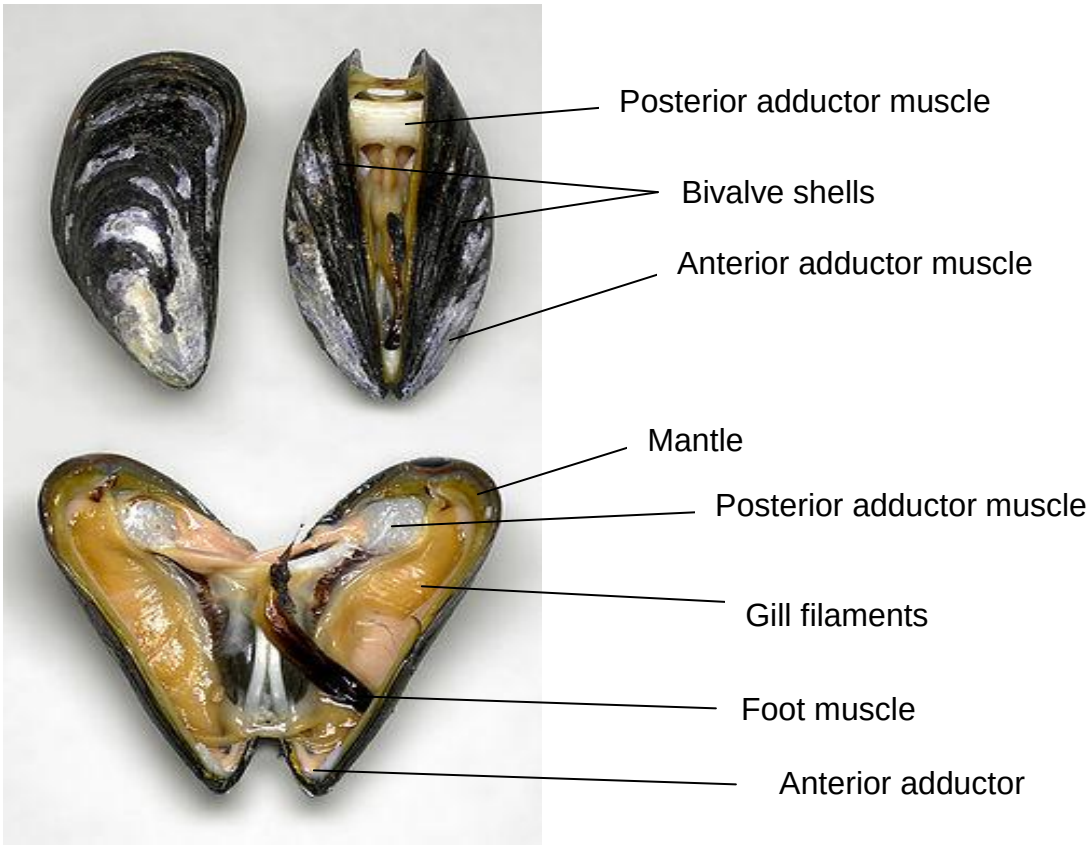


Figure 3.1. Internal structures of Mytilus edulis.

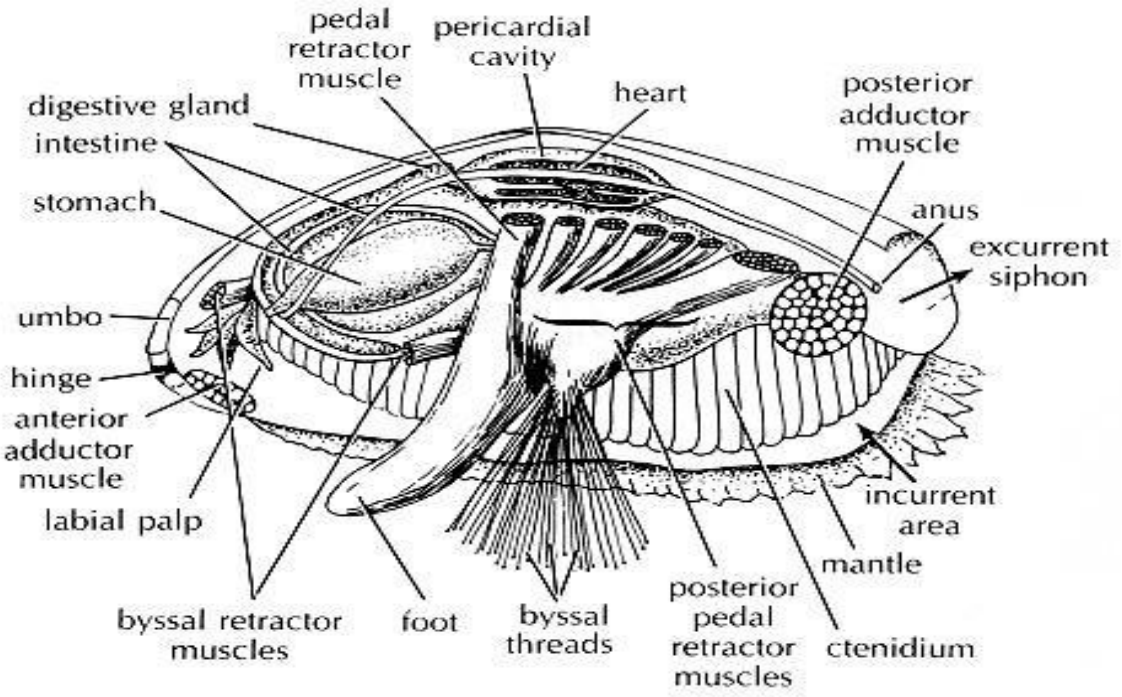


Figure 3.2. Schematic diagram of the internal structures of Mytilus edulis.

3.1.2. Ecotoxicology of lindane

Lindane is a broad spectrum insecticide, although it is now not extensively used throughout the developed world it does show persistence in soils and is frequently found in aquatic systems as a consequence of soil leaching (Villanneau et al., 2009). Lindane has been reported at mean concentrations of 150 ng L^{-1}, peaking to 300 ng L^{-1} (Cole et al., 1984), in China at a mean of 100 ng L^{-1} peaking to 860 ng L^{L-1} (Gao et al., 2008) and peaks of 0.013 µg L^{-1} in the Thames Estuary (Power et al., 1999). Once in the aquatic environment, it can affect communities such as zooplankton, benthic macroinvertebrates and fish. It has been shown to be toxic to both benthic and epibenthic organisms, where (Taylor et al., 1991) found lindane to be the most toxic to both *Chironomus riparius* and *Gammarus pulex* compared with copper, 3,4-dichloroaniline and atrazine. Levels as low as 50 ppb had an effect on the migratory pattern of *D. magna* observed using a behavioral bioassay that monitored their spatial orientation towards light (Goodrich and Lech, 1990). The acute toxicity of lindane to *D. magna* was found to be much higher in the presence of contaminated algae, suggesting the exposure route via food is of significance for daphnids (Fliedner, 1997). Furthermore, the effects of low concentrations of lindane in *Daphnia* have also been observed at the metabolic level, where perturbations of carbohydrate metabolism were investigated using 48–96 h LOEC and EC_{10} values of lindane and mercury (De Coen *et al*., 2001). Both toxicants increased glycolytic and hexose-monophosphate activity and lindane inhibited cellular lactate formation with increased Kreb's cycle activity after 48 hours. Catalase activity, a biomarker of

exposure to an oxidative stress, was measured in the tissues of the marine mussel (*Mytilus galloprovincialis*) (Khessiba et al., 2005). Mussels were treated with lindane at a concentration of 40 µg L^{-1} and catalase activity increased compared with the controls; this was similar to effects exhibited in response to high temperature, high salinity and increased light intensity. Catalase activity decreased when food, was available in controls, whilst food supply did not change catalase activity to those exposed to lindane further supporting the deleterious effects of lindane exposure.

3.1.3. Ecotoxicology of atrazine

The triazine herbicide atrazine is widely used for the control grasses and weeds in many crops and other non-agricultural applications. A high runoff potential means that atrazine is frequently detected in aquatic ecosystems and its concentration can exceed values of 9 to 25 µg/l in Europe (Croll, 1991) and 87 to 100 µg/l in North America (Graymore et al., 2001) with a level of 20 µg/L regarded as a reasonable no observed effect level on an aquatic ecosystem (Wilfried, 1993). Although here in the UK levels in the Thames Estuary were significantly lower peaking at 0.1 µg L^{-1}(Power et al., 1999). In the European Union, atrazine has been withdrawn from use because levels in groundwater widely exceeded the statutory maximum concentration for any single compound in ground water (Pavlis et al., 2010). However, it is still the most widely used herbicide in USA.

Due to its persistence in aquatic environments, atrazine and its metabolites can cause sublethal effects in various aquatic organisms. Since its mode of action inhibits photosynthesis, its most direct effect is on the algal community. This in turn will have a knock on effect on the grazers of the phytoplankton (Graymore et al., 2001). Further studies on the effect of atrazine on other aquatic animal species have found some direct toxicological effects. The freshwater snail *Lymnaea palustris* was used to characterise an immunological response to long-term (28 days) exposure to a high concentration of atrazine (24 mg/L) (Russo and Lagadic, 2000), where hemocyte density, and phagocytic activity and associated oxidative burst were shown to be responsive to atrazine. At lower concentrations the herbicide exhibits both synergistic and anatagonistic effects when in combination with other pesticides. Atrazine alone did not show significant toxicity to the larvae of *Chironomus tentans* up to concentrations of 1000 µg L^{-1} over 48 hours. However concentrations as low as 1 µg L^{-1} in combination with dimethoate at EC_{25} significantly enhanced the OP's toxicity, whilst reducing the toxicity of omethoate (the analogue of dimethoate where the sulphur is replaced by oxygen)(Anderson and Zhu, 2004). Mixtures of atrazine and diazinon also displayed increased toxicity to the daphnid *Ceriodaphnia dubia* with atarzine concentrations of 5 µg L^{-1} with the LC_{50} of diazinon falling from 0.21 µg L^{-1} when used alone, to 0.16 µg L^{-1} in combination with atrazine (Banks et al., 2005), with both concentration levels found occurring within environmental waters.

3.2. Aims

- To investigate the impact of individual toxicants in the aquatic organism *Myitilus edulis* by two model toxicants, atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-,3,5-triazine-2,4-diamine) and lindane (1,2,3,4,5,6-hexachlorocyclohexane) using NMR spectroscopy coupled with metabolite addition experiments to identify the major peaks within the metabolic profile of the test organism.
- To optimise the extraction technique of the small molecular weight metabolites from the tissue matrix of *Myitilus edulis* with minimal interference from proteins and lipids and identify the intraspecific variation between the control test organisms under steady state conditions.
- To investigate the metabolic perturbations of the environmental stressors hypoxia and starvation and compare these to the effects of the toxic insults.

3.3. Materials and methods

3.3.1. Chemicals and reagents

Lindane (97% γ-isomer) and atrazine (pure analytical standard), and amino acids were purchased from Sigma Aldrich (Poole, UK). All solvents (chromatographic grade), salts (Analar grade), and acids (Analar or better grade) were obtained from Fisher Scientific UK Ltd. (Loughborough, UK). Deuterium oxide (99.6% (v/v)) was purchased from Goss Scientific Instruments Ltd (Nantwich, UK). All of the water used in the laboratory was Purite de-ionised water. Bond Elut® C_{18} solid-phase extraction cartridges were obtained from Varian Ltd. (Oxford, UK). Artificial sea water salt mix (Red Sea) and artificial diet (Kent Marine Phytoplex) for the mussels was obtained from Maidenhead Aquatics (Havant, UK).

3.3.2. Mussel culture

Mussels were brought from Solent Fish (Portsmouth) and sorted by size and those in the size range of 50-60 mm were kept for test purposes. The mussels were sourced from Scottish waters and were rope-grown offshore; therefore the culture was of a similar age and showing the same number of seasonal ring features on their outer shells. The shells of the mussels were cleared of any encrusting organisms using the back of a knife under running water. The mussels were then transferred to a holding tank that had a continual flow of Langstone Harbour seawater supplying the organisms with natural food,

oxygen (> 80 % saturation) and removal of waste material. The salinity was relatively stable at 34 ‰, although the water temperature varied with the environmental fluctuations (7-23 °C) throughout the season and organisms were also exposed to a natural photoperiod. An occasional cleanout was implemented every week to remove the build up of sediment that may cover and eventually suffocate the organisms.

Before the organisms' were used in any experimentation they were moved to a different holding tank inside the marine laboratory and a stable temperature was maintained at 15 °C using water heaters for a two week period before testing. The seawater was a flow-through system that was filtered to remove particulate matter; therefore they were feed on an artificial diet of planktonic substitute (Phytoplex, Kent Marine, Wisconsin, USA). This was also used during testing. The holding tank was also cleaned daily by draining the water to remove any faecal matter that built-up. This also allowed the organisms to acclimatise to the water removal that occurred daily during experimentation using a semi-static renewal system. The organisms were fed after the cleanout procedure and left for several hours before filtration was resumed to allow feeding without loss of the food particles.

As these organisms display a seasonal spawning event when the animals deplete the energy stores built-up during the year and become spent. The animals used in this study were investigated in the early winter months during which gonad production is at an early stage and it is difficult to determine the

gender of the animals, therefore in this study the sex of the test animals was not known.

3.3.3. Species identification using polymerase chain reaction of foot protein gene sequences

3.3.3.1. Variation of adhesive protein

Due to the morphological similarity and overlapping populations of *Mytilus edulis* and *Mytilus galloprovincialis* found in the UK (Hilbish *et al*., 2002), it is extremely difficult to differentiate between the species by morphology alone (McDonald *et al*., 1991). The subtle differences are indicated as *M. galloprovincialis* attaining a slightly larger size and inhabiting the top of the littoral zone, with the umbones turning downwards giving a concave appearance. These characteristics can be further hindered by morphological changes due to local environmental conditions, with changes in substrate and wave energy leading to subtle changes in shell formation (Gosling, 1992), increasing the difficulty in formal identification. It was therefore important to identify another strategy to determine the species of the test population of *M.edulis.* For this a subsequent set of mussel samples was collected to from two sites, *M. edulis* sourced from East Sands, St Andrews, Fife (grid reference: NO 520 164 GB) and a representative species of *M.galloprovincialis* was collected from Woolacombe, Devon (grid reference: SS 455 440 GB).

A variation in an adhesive protein gene sequence of these two species has been identified, isolated and analysed using a polymerase chain reaction (PCR)(Inoue *et al*., 1995). Using copies of the sequenced set of oligonucleotide primers (Me 15 & Me 16 from Promega, Southampton UK) it was possible to distinguish between the two species to confirm the test species identity.

3.3.3.2. Extraction of DNA

The sample mussels were opened with a knife and ~3 mm^3 of adductor muscle was removed using a scalpel. This material was macerated with a small quantity of sterile sand in a lysis buffer (500 μL of 50 mM Tris-HCl pH 8.2, 10 mM EDTA, 0.5 % SDS, 500 μg mL^{-1} proteinase k) and heated to 55 °C for 4 h in a thermomixer (Eppendorf, Comfort, Cambridge, UK). DNA was extracted from the muscle tissue with a phenol:chloroform:isoamyl alcohol (25:24:1 ratio v/v/v) mixture (500 μL) and spun in a centrifuge for 4 min at 10 000 rpm. Chloroform (500 μL) was then added and the sample spun again, the aqueous phase was then precipitated with 1 mL ethanol (100 %) and 40 μL of 3 M NaAc (pH 5.6). The samples were then frozen at –20 °C for at least 30 min and then spun at 13 000 rpm for 15 min. The supernatant was then carefully removed to leave the pellet of DNA. This was washed three times with 500 μL of 70 % alcohol with a 4 min spin at 10 000 rpm between each step and the alcohol removed to leave the extracted DNA sample.

3.3.3.3. Clean up of DNA and gel shift

Before a PCR of the tissue samples could be performed it was important to check the samples for DNA content, as without this the PCR will not work properly. The presence of RNA will also significantly affect the result, as this will block the DNA regeneration process. Therefore a gel shift was performed on the extracted samples to identify the DNA/RNA content. A 2 % agarose gel was made up with 2 g 100 mL^{-1} of 1xTBE and 5 μL of EtBr, the gel powder was heated in a microwave for a few minutes until the liquid was clear, and was then left to set in gel mould. Before the gel test 2 μL of each sample was tested using a NanoDrop Spectrometer (Therm 1000, Fisher Scientific UK) for DNA content, all samples contained DNA, but also significant levels of RNA that needed removing as they inhibit the PCR process. The gel shift ran for 40 min (120 V, 400 mA) containing 2 μL of sample with 2 μL of orange dye and 6 μL of pure water (Promega, Southampton, UK) added to lanes 1-10, one sample in each. A negative control was used with pure water only and a positive control containing DNA marker (50 bp ladder, Promega, Southampton, UK). Unwanted RNA in the samples was digested during a cleanup step. Each sample had 10 μL of buffer 3 (Sigma) and 3 μL of RNAse A and were mixed carefully before incubation at 37 °C for 2 h. Once this was complete the phenol:chloroform and ethanol cleanup steps used above were repeated to leave only the extracted DNA.

3.3.3.4. PCR amplification

Using the set of primers, Me 15 & Me 16 (Invitrogen, Paisley, UK) from Inoue *et al.* (1995), the untranslated 5' non-repetitive region of the polyphenolic adhesive protein gene was amplified by PCR. The primers were reconstituted with 1 mL of pure water giving a nominal concentration of 25 pmol μL^{-1}, along with a positive control containing DNA and a negative control with pure water (Promega, Southampton, UK) only. A test set of ten tissue samples was analysed from the stock population used during experimentation. For the PCR a total of 50 μL of each sample was needed, containing 1 bead of Taq (TaqBead^{TM} Hot Start Polymerase, Promega, Southampton, UK), 5 μL Taq buffer ($MgCl_2$ free), 1 μL dNTP mix (10 mM), 3 μL $MgCl_2$, 2 μl of upward primer (Me 15) and 2 μL of downward primer (Me 16), 35 μL of pure water and 2 μL of sample. Each sample was added to the PCR unit (Storm, GRI, Braintree, Essex) programmed to run 30 cycles of amplification with each cycle consisting of 30 s at 94 °C, 30 s at 56 °C and 90 s at 70 °C and an initial denaturation and final extension periods of 4 min (Inoue et al., 1995, Wood et al., 2003a). A 2 % agrose gel shift (see above) was then run using the PCR products (2 μL) of each sample including a negative control (pure water only) and a positive control containing DNA and a DNA marker (50 bp DNA ladder, Sigma Aldrich).

3.4. Procedures for pesticide exposure

For experimental exposures (30 days), under a static renewal regime (Ernst, 1977), specimens were transferred to glass beakers (2 L) containing artificial sea water (salinity 35 ‰) and maintained at 15°C (16 h light:8 h dark) in a temperature controlled room. Two mussels were used in each tank, and were fed daily with 200 μL of phytoplanktonic food. This was added at two time intervals 4 h apart to keep the level of food in the water above zero percent as it has been shown that mussels will reduce their filtration rate significantly if the organisms have little food particles to filter from the water column (Hawkins *et al*., 1998). The artificial sea water containing each treatment was renewed daily. Two concentration regimes (a high sub-lethal dose and a no effect dose) were used for each pesticide. For lindane these were 1 mg L^{-1} and 5 μg L^{-1}, and for atrazine 3 mg L^{-1} and 5 μg L^{-1}. Stock solutions of lindane (1 g L^{-1} and 5 mg L^{-1}) and atrazine (3 g L^{-1} and 5 mg L^{-1}) were prepared in acetone and stored in the dark at -20 °C. Fresh incubation solutions in sea water were prepared from the stock solutions daily for replenishment of the beakers. These working concentrations of the pesticides were produced by the addition of 2 mL of the appropriate stock solution in acetone to 2 L of sea water in order to maintain a constant concentration (1 mL L^{-1}) of acetone. The acetone was used to aid solvation and ensure uniform distribution of the test chemicals in the tank. Controls were exposed to the same concentration of acetone alone. Artificial sea water used for the replacement solutions was cooled and held at 15 °C for at least 48 h before use. The spent artificial sea water was filtered through activated charcoal before disposal. In the

experiment to investigate the effects of lindane, eight mussels were allocated to each treatment (control, high dose, and low dose). For atrazine seven mussels were allocated to each of three treatments (control, high dose, and low dose), and five to the other two treatments (hypoxia, and starvation). The numbers of animals used was determined by the space available in the constant temperature facility, and there was no mortality during the experiment.

High, sub-lethal doses of lindane (1 mg L^{-1}) and atrazine (3 mg L^{-1}) were equitoxic (0.5 LC_{50}) and were selected on the basis of earlier work by (El-Shenawy *et al*., 2006, El-Shenawy *et al*., 2003) that demonstrated a clear impact on the metabolism, physiology and behaviour of *M. edulis*. Lower doses (lindane 5 µg L^{-1}, and atrazine 5 µg L^{-1}) were in the region of reported no effect concentrations in chronic exposure to lindane (daphnid and fish) and much lower than those for atrazine (chironomid and fish) (Girling et al., 2000). These levels were designed to investigate effects on metabolism in the absence of gross externally detectable toxicological modifications of physiology and behaviour. Use of these model pesticides has been heavily restricted in many countries in recent years, and as a consequence both of these concentrations are higher than those found currently in surface waters including estuaries in many regions of the world (Graymore et al., 2001). Hypoxic conditions were imposed by exposing mussels to air for 10 h prior to tissue extraction, and for the starvation treatment animals were left unfed for 10 days prior to sacrifice. Water quality (pH, oxygen and ammonia concentrations) was monitored throughout the exposure period, and remained

constant. Nominal concentrations of the pesticides were checked regularly by analysing aliquots (1 L) of the exposure media. A set of glass tanks used in the exposure of the mussel samples were set up at 15 °C containing 1 L of artificial seawater (Red Sea). A set of dilutions from the 10 000 ng mL^{-1} stock solution were made to 5 000 ng mL^{-1} for both lindane and atrazine these concentrations were added to two sets of artificial seawater and left for various time intervals before removal by solid phase extraction (SPE). The time intervals were 0, 1, 2, 4, 8, 12 and 24 h for both toxicants to observe the loss of compound through photo-degradation, evaporation and adsorption to the glassware and other preparation and extraction materials. After the toxicants were added and thoroughly mixed the samples were removed at the desired time interval and the solution was transferred to a darkened bottle and sealed to stop further degradation. These solutions were then extracted through a SPE (C_{18} Varian, Oxford, UK) column using a pump system. Each column was first activated by the addition of 1 mL of methanol, then HPLC grade water was added to stop the column from drying out while the seawater samples are set up. This is achieved by connecting 5 mm Teflon tubing from the bottle through a bung to keep the samples sealed and connected to the SPE by another bung to conserve the vacuum. The tap was opened and the vacuum provided by the pump pulls the seawater through the SPE column where the compounds are retained on the column whilst removing the water to a waste vessel.

Once the water sample has passed through, each SPE column was left open to the air with the pump still running to allow the column to dry. Once dry the

cartridges can be stored at –20 °C until needed so that all the samples can be extracted before being eluted together ready for analysis. The compounds were eluted from the SPE columns using appropriate solvents added to the cartridges. The elution solvents used were *n*-hexane for lindane and methanol for atrazine, 1 mL of either solution was added to the thawed cartridges and allowed to drain into GC/MS vials labelled according to the sample time. To make sure as much of the solvent was eluted through the column into the vials a 20 mL syringe was used to provide back pressure through the column to aid elution. The vials (tinted brown to reduce further photo-degradation) were then analysed using the GC/MS and a sequence set. Vials were set up randomly to reduce any potential bias, with extended clean up steps using a toluene needle wash followed by a vial containing elution solvent only, between each sample as the high concentration of the toxicant was found to carry over to the next sample without these extra clean up procedures.

Extracts were analysed using standard GC/MS methods with single ion monitoring (atrazine m/z = 173.10, 200.10, 215.20, and lindane m/z = 111.10, 181.00, 219.00). The levels of both pesticides fell over the 24 h periods between replenishment both in control (with no mussels) and test beakers. For lindane the concentration fell to between 63-71% (fig. 3.3) of the nominal levels in the absence of animals and to 47-64 % where mussels were present. The equivalent figures for atrazine were 68-75 %, and 61-69 % respectively (fig. 3.4). These losses could be attributed to adsorption to the glass surfaces and particulate matter, evaporation and photo-degradation as well as uptake by the mussels. It has been shown previously (El-Shenawy *et al*., 2006) that

under similar exposure conditions the mussels bioaccumulated significant amounts (concentration on a wet weight basis in foot tissues of 13 µg g^{-1} of atrazine, and 60 µg g^{-1} of lindane after 28 days exposure).

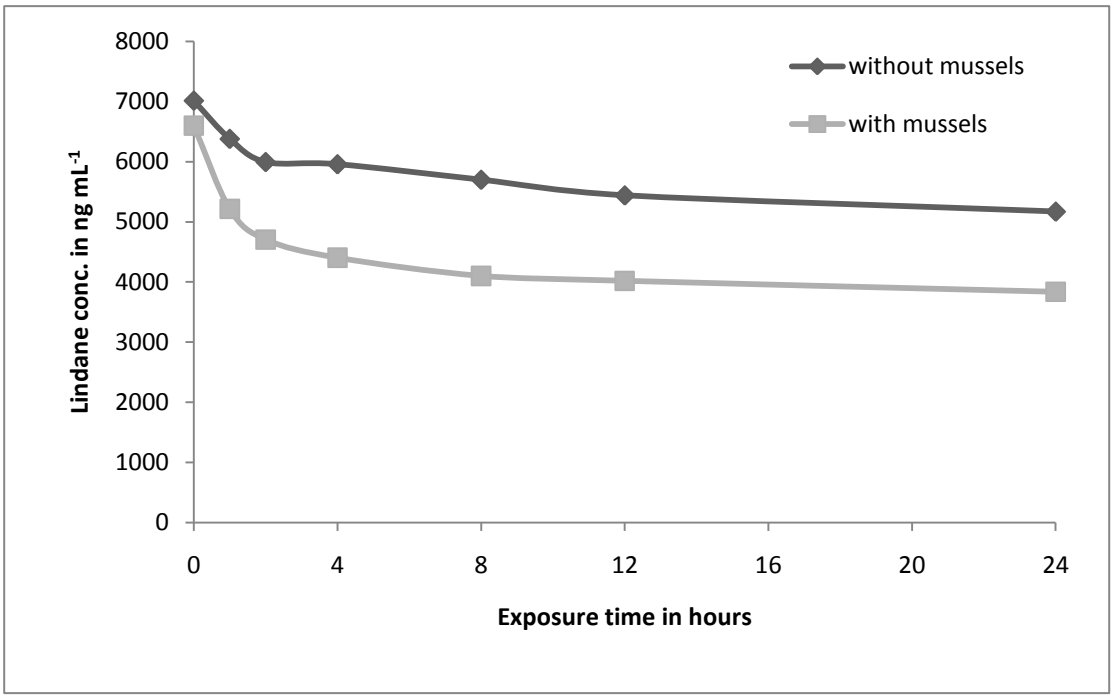


Figure 3.3. Concentration of lindane in test water over 24 h exposure period with and without mussels present.

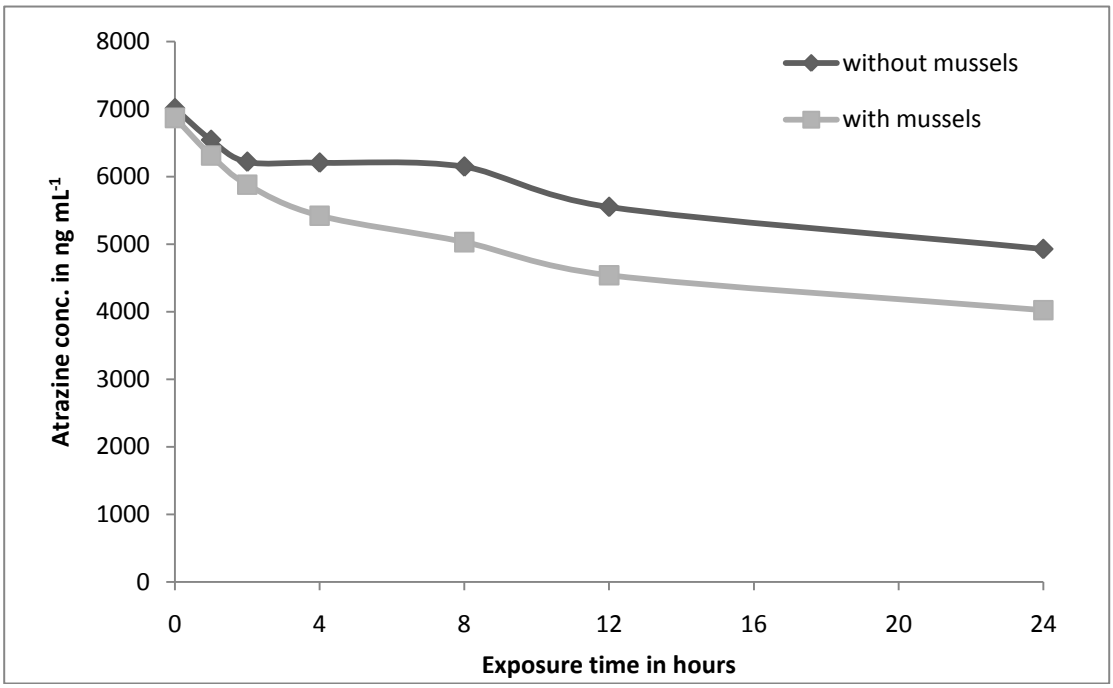


Figure 3.4. Concentration of atrazine in test water over 24 h exposure period with and without mussels present.

3.4.1. Behavioural observations of *Mytilus edulis* exposed to lindane and atrazine

The ability of bivalve molluscs to accumulate toxicants has led to the use of these organisms as biological monitors of their surrounding environments (Kramer *et al*., 1989). The use of bioaccumulation on its own as a warning of contamination has the distinct disadvantage that time is needed for the concentrations to build up. Physiological and behavioural reactions in contrast are rapid direct responses to the environmental conditions and overall organism health (Widdows et al., 2002). Thus during this study the behavioural responses of the test organisms were monitored throughout the dosing procedure, with the organism health used as a measurement of the chemical stress of the exposed mussels. A preliminary study was conducted on the two high dose levels of the toxicants used (1 mg L^{-1} lindane, 3 mg L^{-1} atrazine), a 14 day exposure was conducted to observe the effects of these high concentration on the survival of the organisms before a full experiment was implemented. Two mussels were stuck to glass slides and added to the 2 L test vessels so they could be positioned at one side of the crystallising dish in front of a digital camera (GZ-MG50, JVC, London, UK). A second dish containing two mussels was positioned parallel to this so that two separate environments (control/exposed) were observed simultaneously. It was then possible to record the mussel valve movements during the exposure period. These were subsequently watched back at speed (x16) and the movements documented. The experiment coincided with the organism's cycle of reproduction and it was observed that over the 14 day period the control

group were in the full process of gametogenesis. Those organisms exposed to the lindane were found to have a significantly reduced gamete production. This would suggest that the stressor had either much reduced or stopped gamete production in those organisms exposed to lindane. There was a less significant reduction in those organisms exposed to atrazine. Due to this it was decided that the full experiment must take place when no significant gametogenesis was taking place as the reduced process in the exposed organisms could display a significant change in the metabolism of the organisms to those of the control group due to this reduction in gamete production. This could then mask any metabolic changes that were occurring due to the toxicological stress of the toxicant alone.

3.4.2. Tissue extraction

Tissue (foot muscle) samples (approximately 1 g wet weight) were taken from the live mussels to be analysed using the method outlined in sections 2.2 and 2.3.

3.4.3. ^{1}H NMR spectroscopy

Spectral acquisition of the samples was conducted using those described in section 2.6.

3.4.4. Spectral pre-processing and statistical analysis

The binning process outlined in section 2.7 was followed to give a non-square matrix for lindane comprising of 24 samples by 671 bins, and for the atrazine, hypoxia, and starvation experiments it comprised 31 samples by 658 bins. The small difference in the number of bins between the two experiments is due to the fitting software that combines (on the basis of the integration slope) adjacent bins that overlap a single peak. The resultant matrix data were then analysed using the statistical analysis outlined in section 2.8.

3.5. Results and discussion

NMR spectroscopy provides a means of obtaining a profile of metabolites present in body fluids or tissues of organisms, and these can be compared between healthy individuals and those subjected to a toxic insult (Bundy *et al.*, 2001). The changes produced in the pattern of metabolites can provide clues as to the secondary lesions caused by various toxicants. These have the potential to identify useful biomarkers of exposure to different classes of toxicants, including industrial chemicals and pesticides. One significant difficulty in this work is separating the effects of specific stressors from natural biological variation and seasonal changes in metabolism (Viant et al., 2003). This background variability can be increased significantly where there are marked differences in metabolite profiles between sexes and closely related species of test organisms (Hines et al., 2007). Despite these difficulties NMR-based metabolomics using multivariate analytical techniques has been used with some success in identifying changes in metabolism associated with biological, chemical and physical stressors in different species (Viant et al.,

2003). This study showed changes in the metabolite profile that are associated with poisoning with the herbicide atrazine, and the pesticide lindane, and with environmental stress (hypoxia and starvation). The latter were studied because the toxicants cause a decrease in rates of feeding and ventilation, and these may mimic the effects of the environmental stressors. The pesticides have been shown to have different modes of action but in this study the two were investigated at different times of year when there were differences between the profiles of control animals. Thus it is not possible to compare the two directly, but only with the appropriate controls.

3.5.1. Identification of mussel species using PCR

The preliminary gel test to determine the sample DNA content before PCR amplification show that samples in lanes 1, 2, 3, 5, 7, 8 and 9 had DNA content (Fig. 3.5). These were saved for PCR, although all samples included amounts of RNA within the samples. The RNA was removed from these samples before PCR (as this inhibits the reaction) with an RNAse clean up procedure.

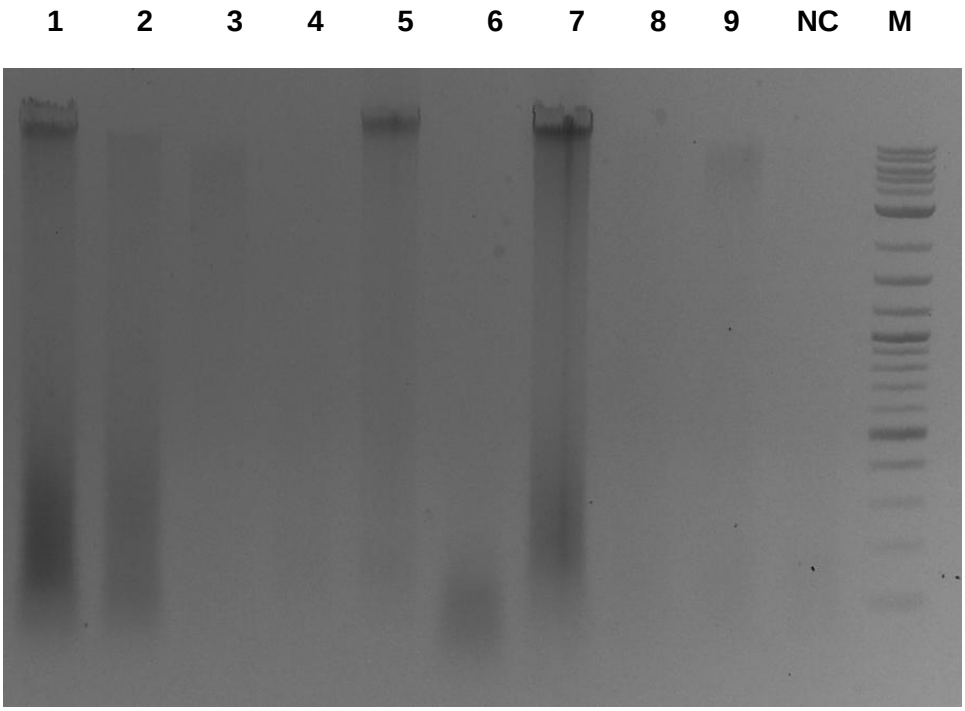


Figure 3.5. Gel shift of DNA/RNA content of tissue samples before PCR of foot proteins, lanes 1-9 indicate tissue samples and NC is a negative control of pure water and M is a molecular marker. DNA is indicated at the top of the gel with RNA at the bottom.

The tissue samples taken from representatives of both species were used to identify the difference in base pair (bp) sequence length between the two species (Fig. 3.6). The band position for each species was constant throughout, but differed between the two distinct species at 180 bp for *M. edulis* and 126 bp *M. galloprovicialis* giving a small but definite separation of 54 bp between shifts. It was therefore possible to distinguish between the two morphologically similar species using this difference in band shift.

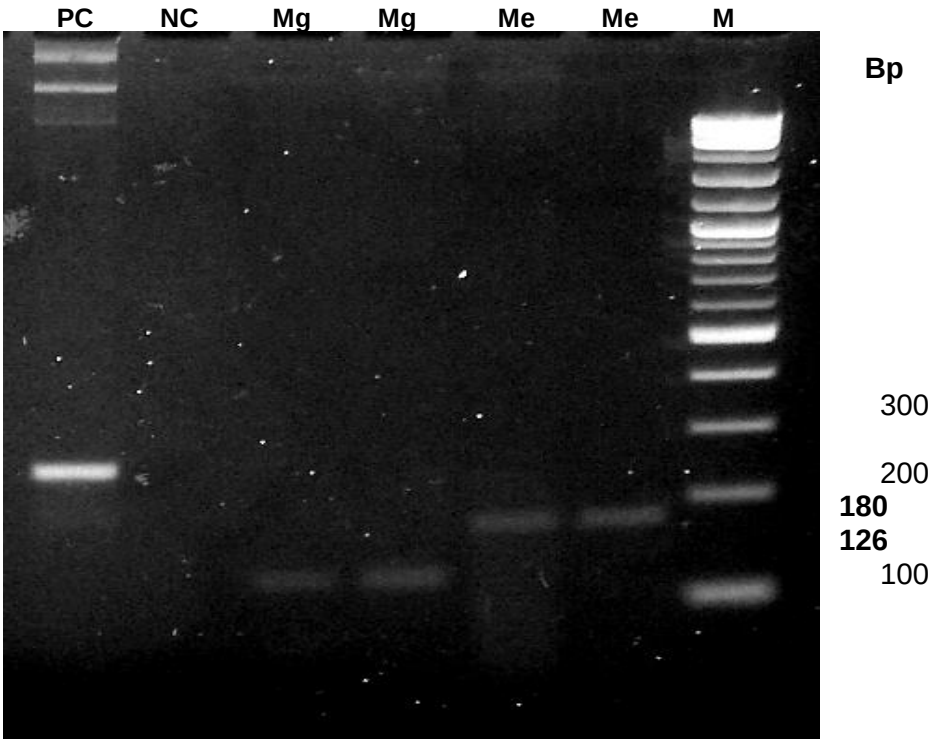


Figure 3.6. Amplification results of the non-repetitive region of the adhesive protein gene. PC is a positive control containing DNA, NC is a negative control containing pure water only, Mg is the protein isolated from *Mytilus galloprovincialis* tissue, Me is the protein isolated from *Mytilus edulis* tissue and M is a DNA ladder with 100 bp spacing.

Lanes 1-10 (Fig. 3.7) representing the tissue samples from the cultured population indicated a clear band shift at 180 bp, although lane 8 was extremely faint due to loss of DNA sample in processing. None of the 10 samples displayed a band shift at 126 bp, as the species of *Mytius trossulus* has been shown to display a band shift at both 180 and 126 bp (Wood et al., 2003). From the PCR and subsequent gel shifts all ten samples were identified as of *M. edulis* and not the invasive continental species of *M. galloprovincialis* or *M. trossulus*. As all the test population came from a

Scottish site it was therefore concluded that the culture population was made up of wholly one species, that of *M. edulis*.

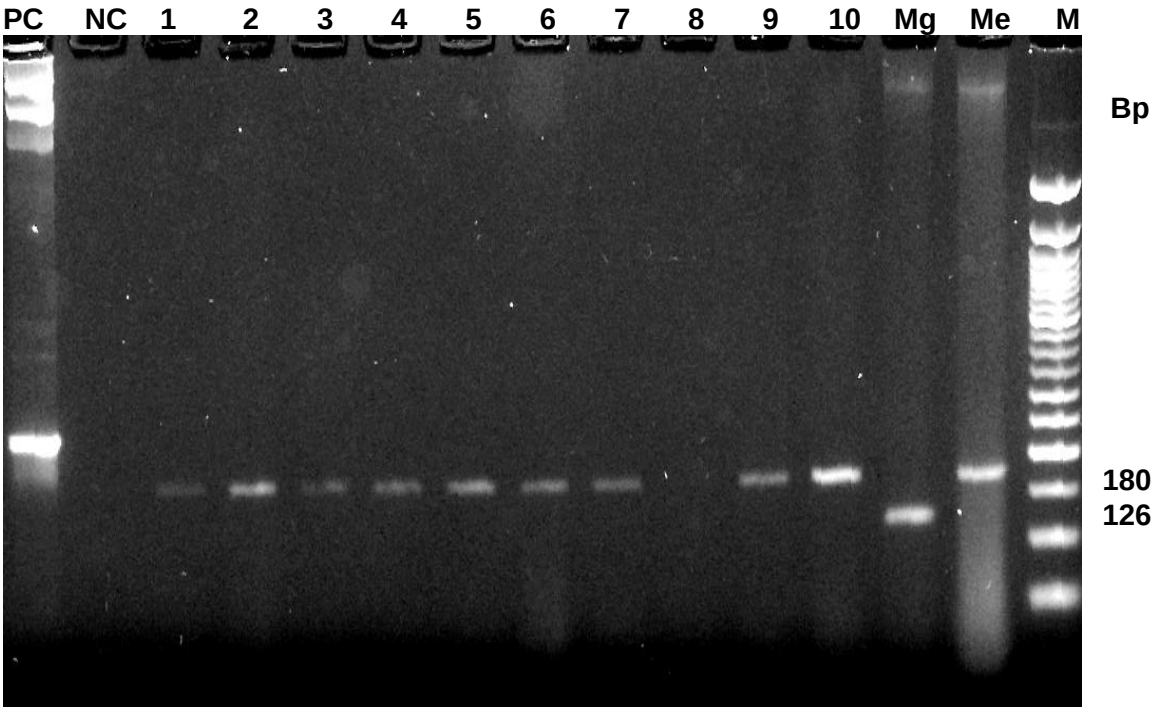
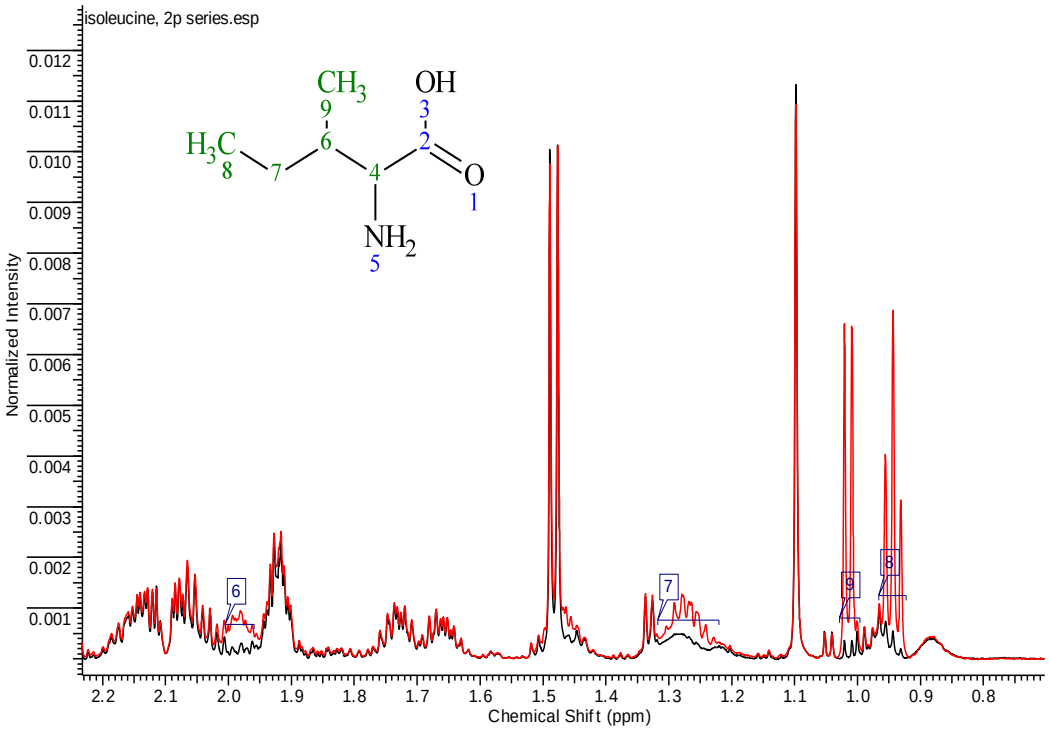


Figure 3.7. Amplification results of non-repetitive region of the adhesive protein gene, PC and NC are positive and negative controls respectively with lanes 1-10 representing products from the randomly selected tissue samples of the test culture mussels. Mg and Me represent products from *M. gallorpovincialis* and *M. edulis* respectively and M is a DNA ladder with 50 bp intervals.

3.5.2. Amino acid fortification experiments

The following set of figures 3.8. a-w shows the results of these fortification experiments. Each spectrum also displays the chemical structure of the metabolite being identified with the protons in the molecule numbered and their position highlighted within that structure.

a



b

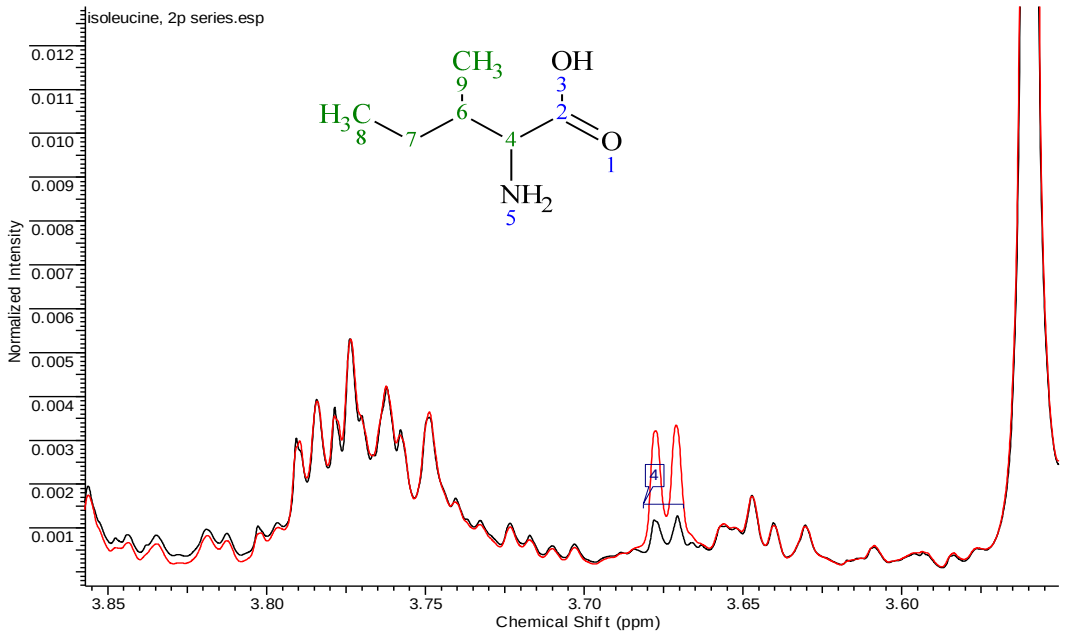
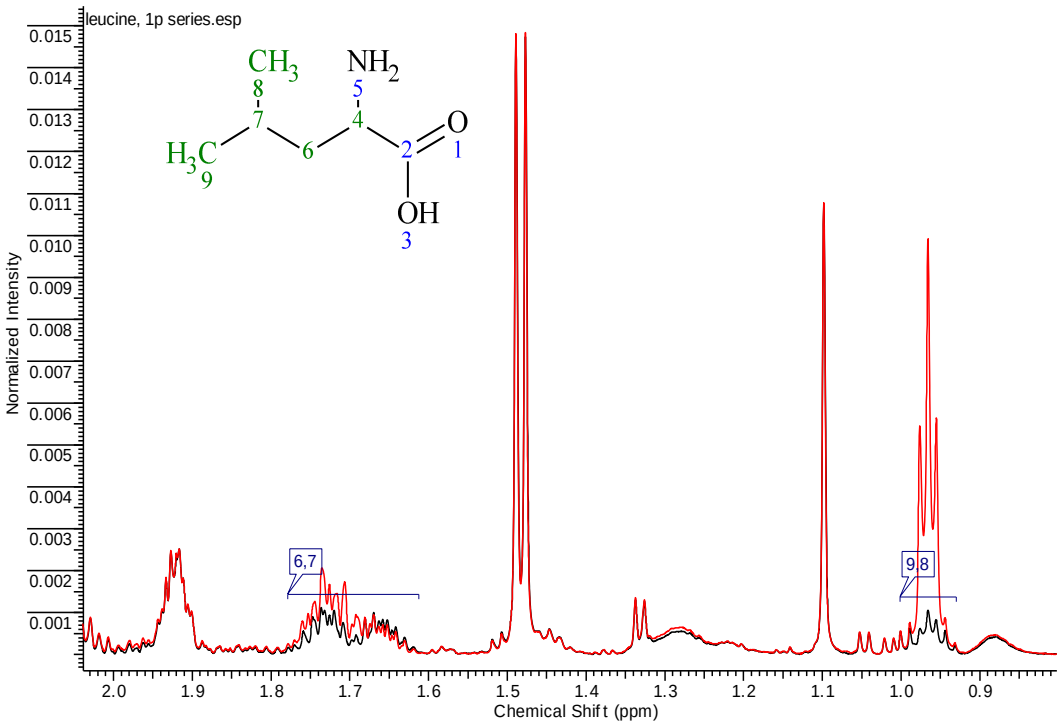


Figure 3.8. a-b. Peak positions of isoleucine after standard addition with corresponding chemical structure. The red line is the spectrum after the addition of isoleucine (5 μg), with the numbered boxes corresponding to the proton highlighted within the structure.

c



d

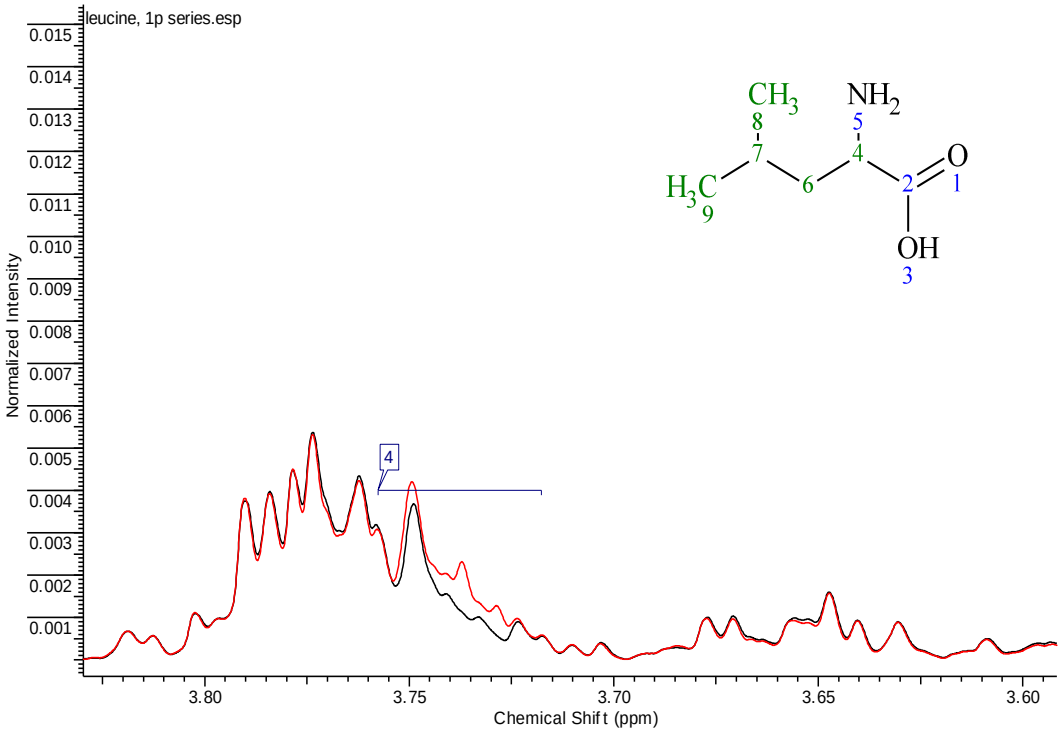
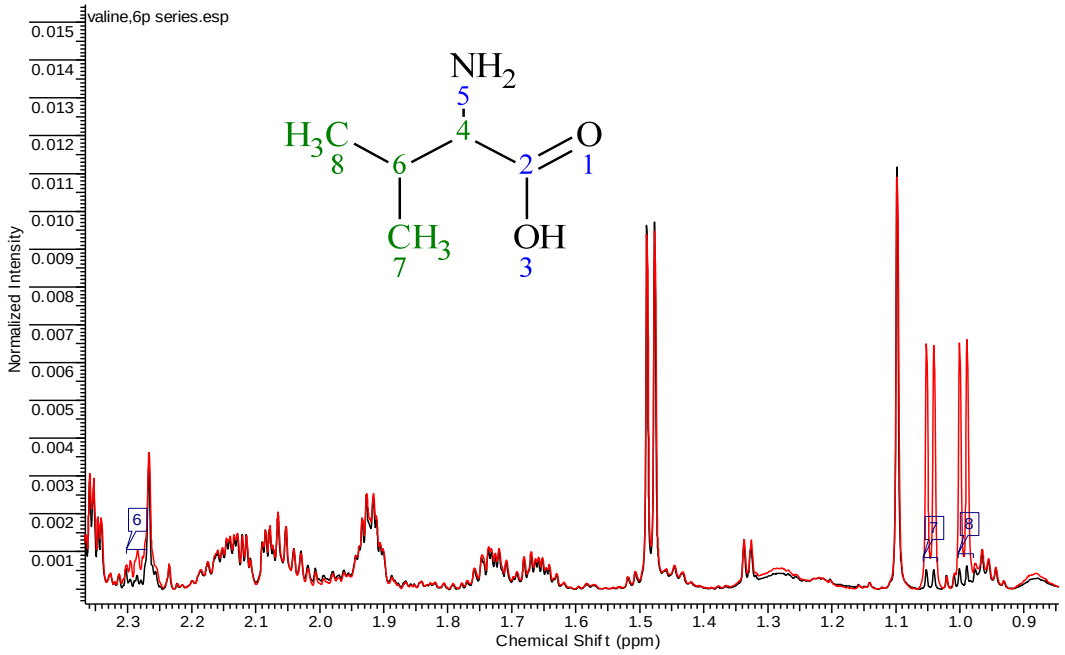


Figure 3.8. c-d. Peak positions of leucine after standard addition with corresponding chemical structure. The red line is the spectrum after the addition of leucine (5 μg), with the numbered boxes corresponding to the proton highlighted within the structure.

e



f

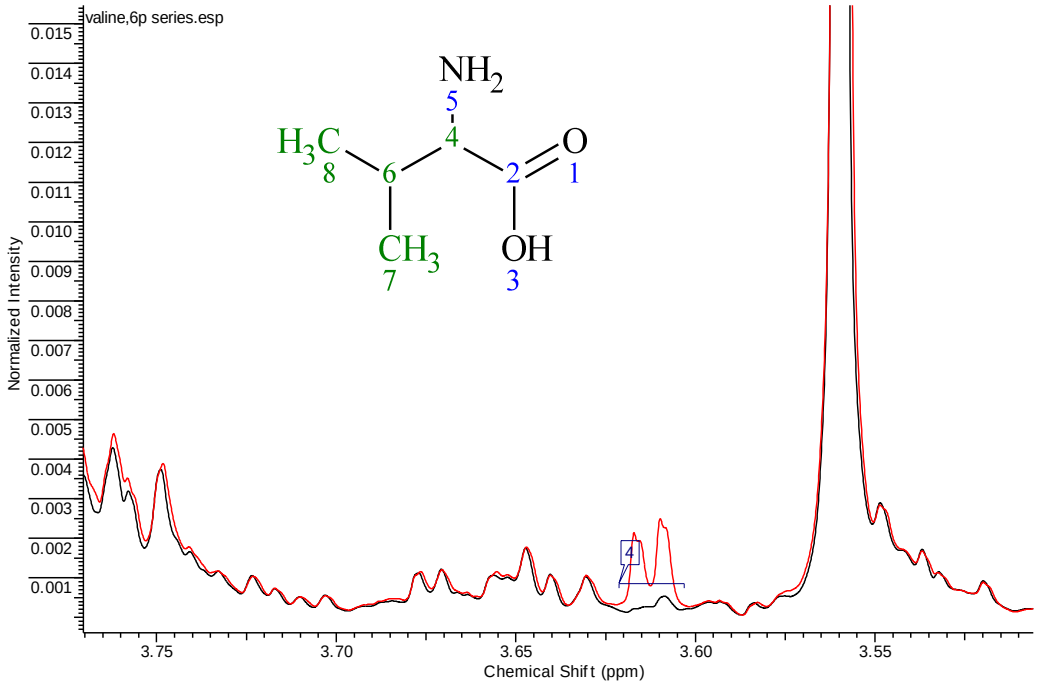
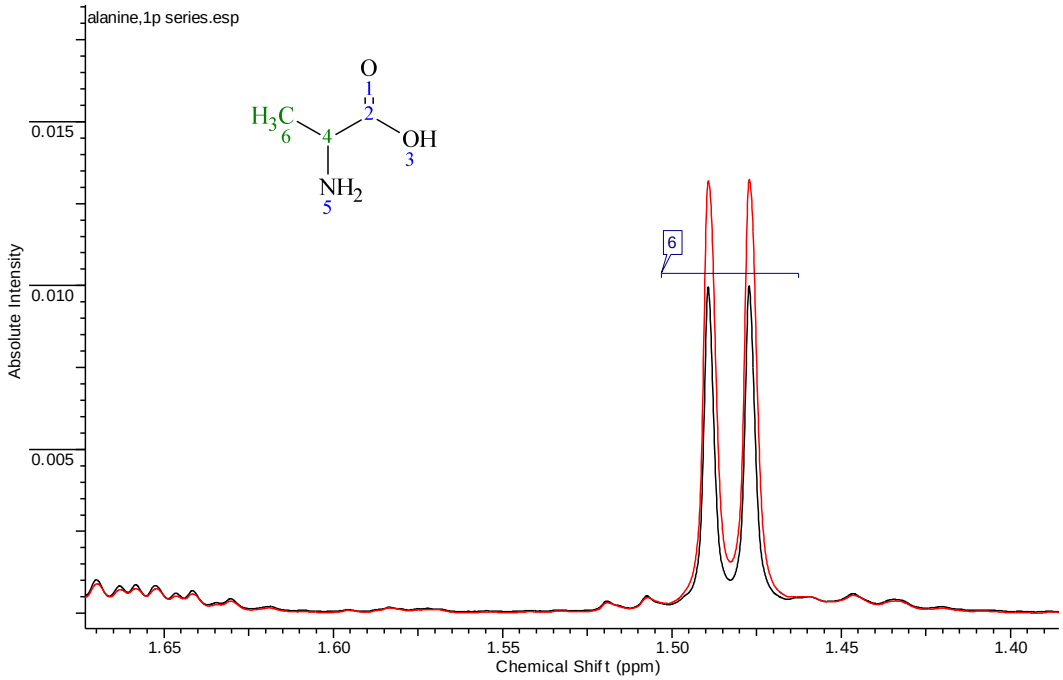


Figure 3.8. e-f. Peak positions of valine after standard addition with corresponding chemical structure. The red line is the spectrum after the addition of valine (5 μg), with the numbered boxes corresponding to the proton highlighted within the structure.

g



h

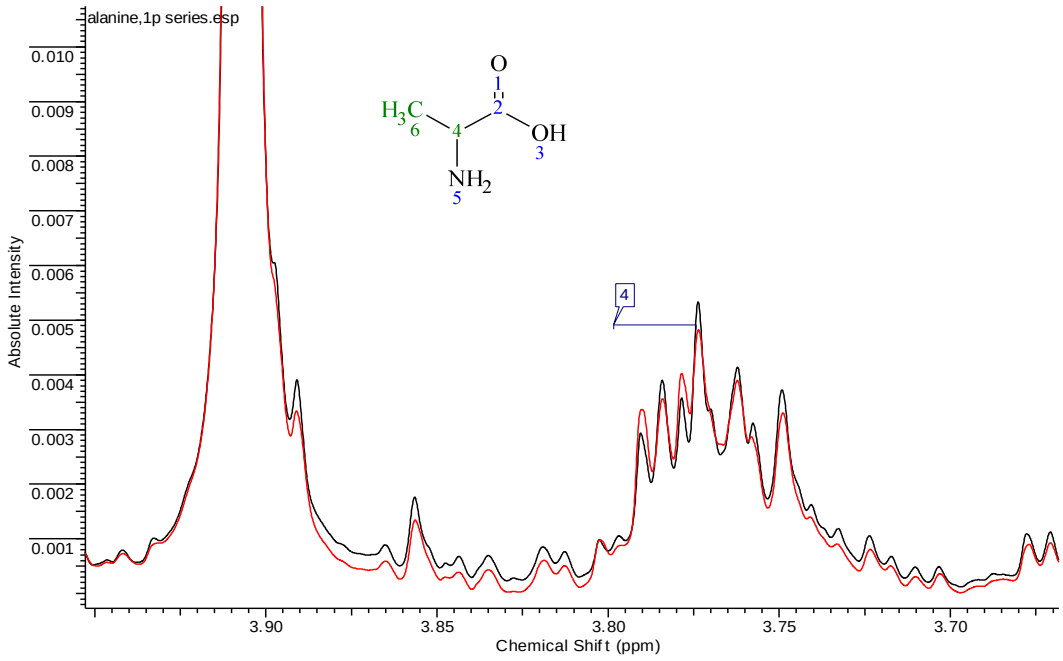


Figure 3.8. g-h. Peak positions of alanine after standard addition with corresponding chemical structure. The red line is the spectrum after the addition of alanine (5 μg), with the numbered boxes corresponding to the proton highlighted within the structure.

i

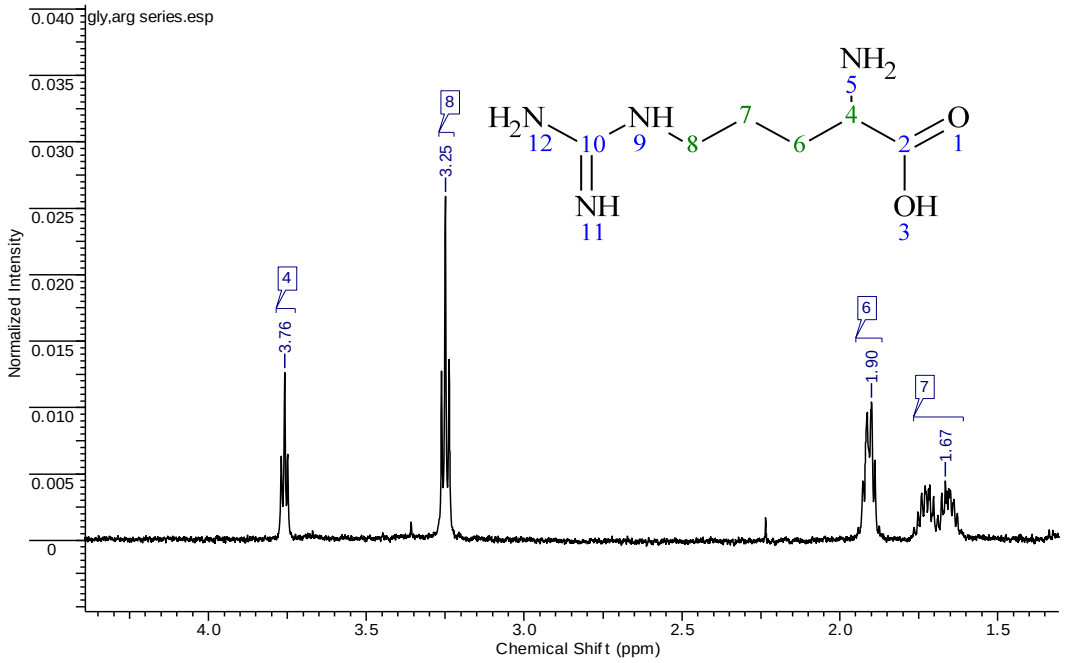
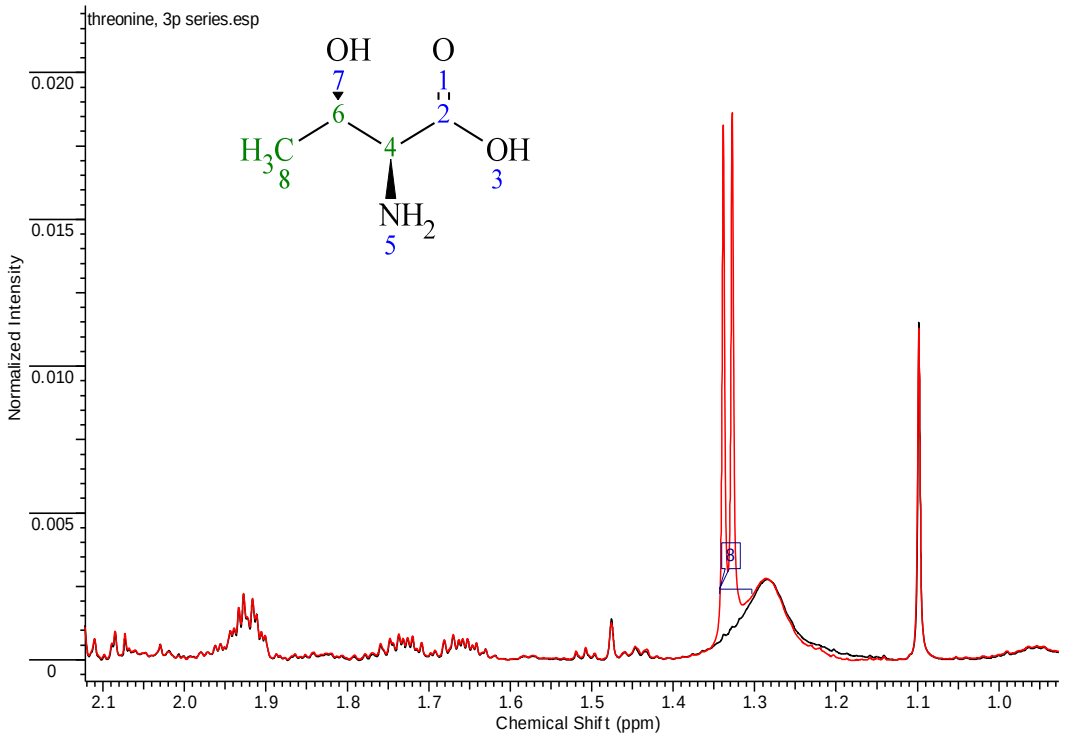


Figure 3.8. i. The spectrum of arginine (5 μg) dissolved in to $^{2}H_2O$, and the corresponding chemical structure.

j



k

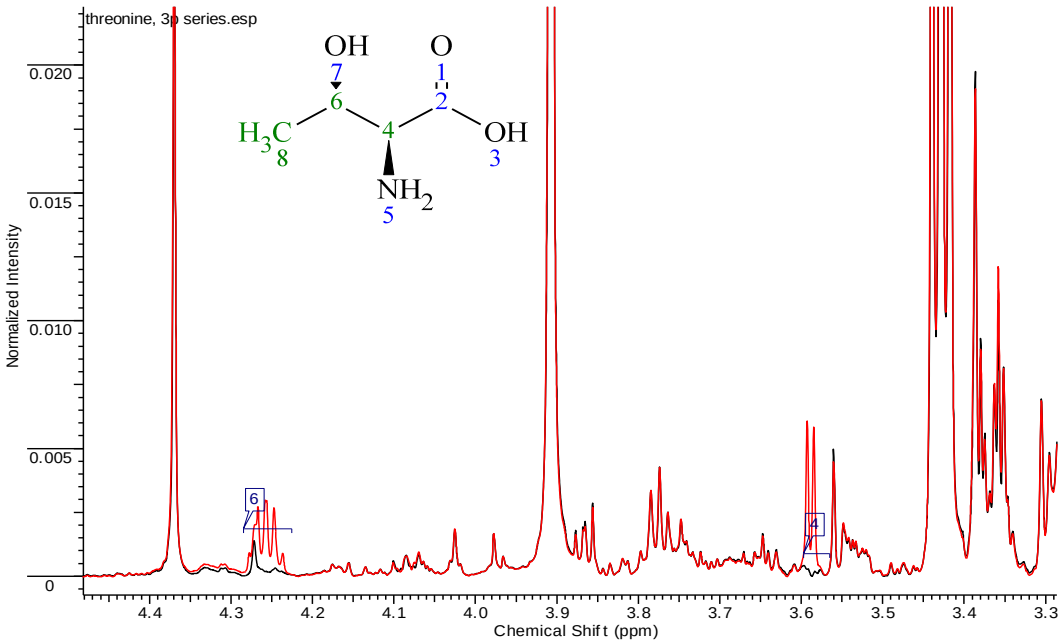
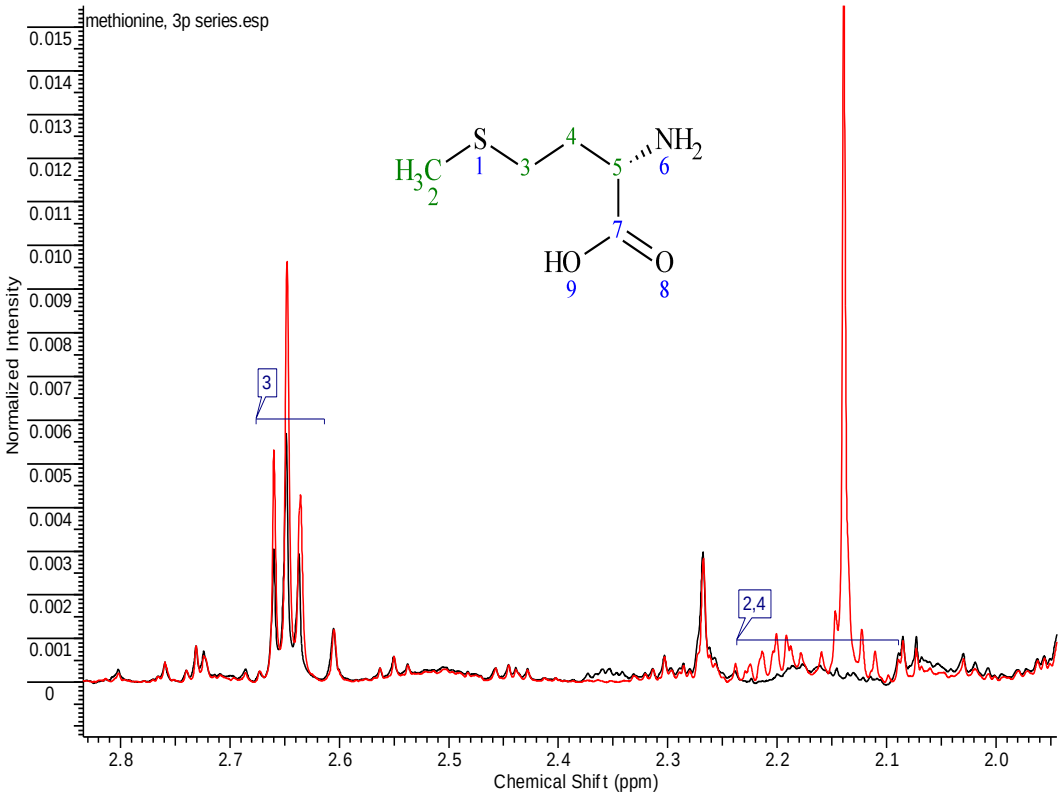


Figure 3.8. j-k. Peak positions of threonine after standard addition with corresponding chemical structure. The red line is the spectrum after the addition of threonine (5 μg), with the numbered boxes corresponding to the proton highlighted within the structure.

l



m

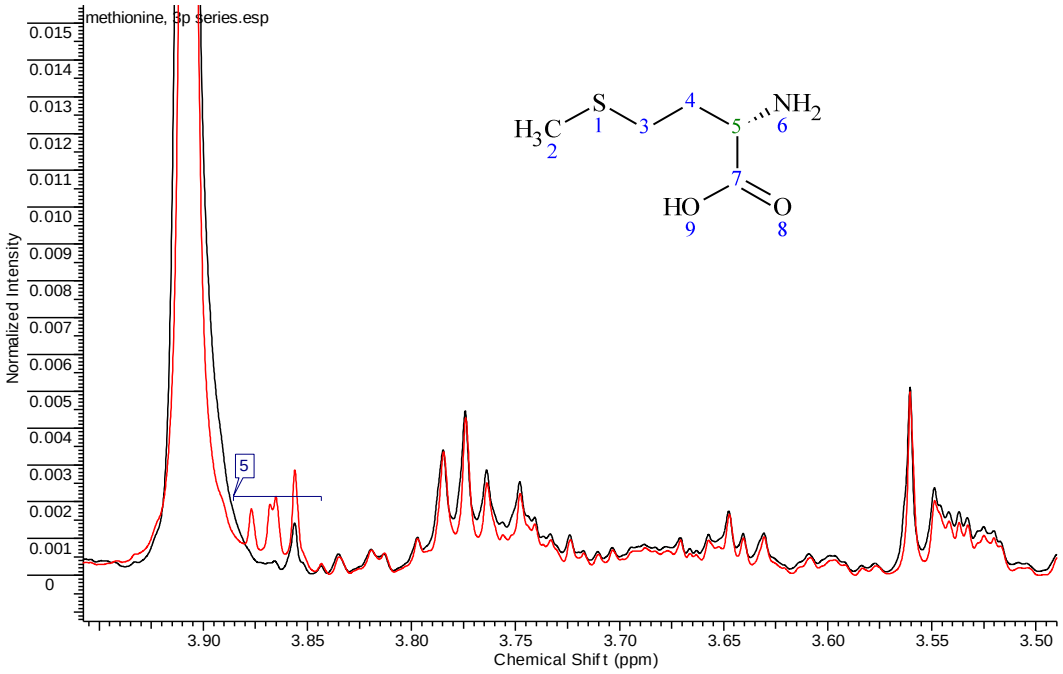
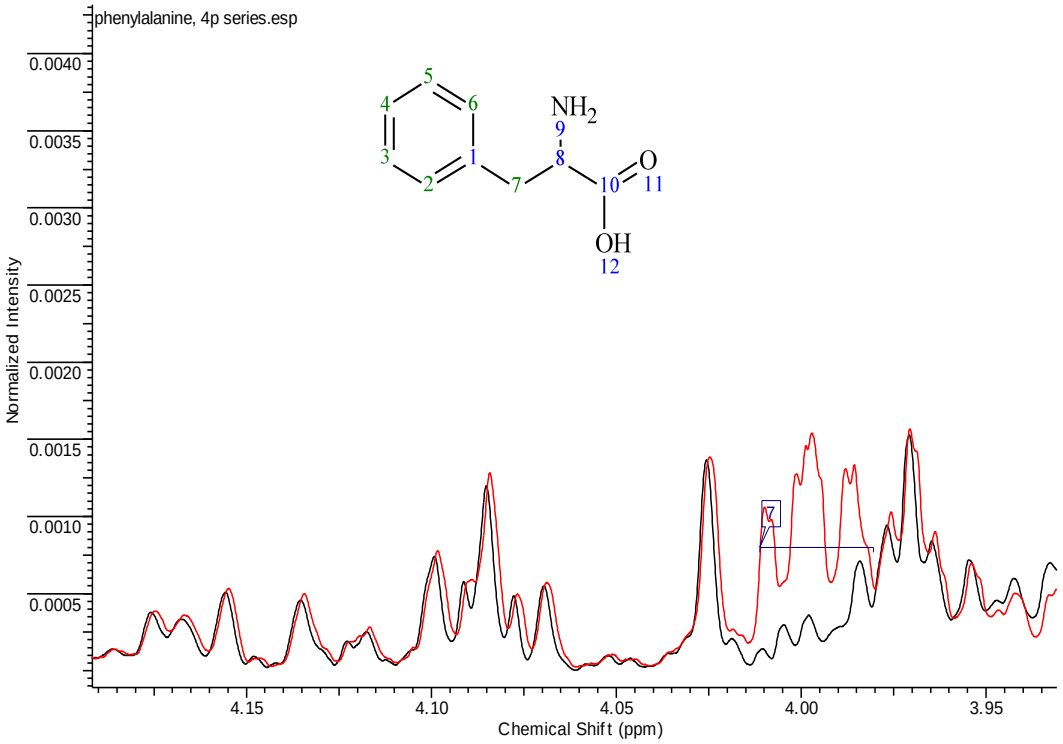


Figure 3.8. l-m. Peak positions of methionine after standard addition with corresponding chemical structure. The red line is the spectrum after the addition of methionine (5 μg), with the numbered boxes corresponding to the proton highlighted within the structure.

n



o

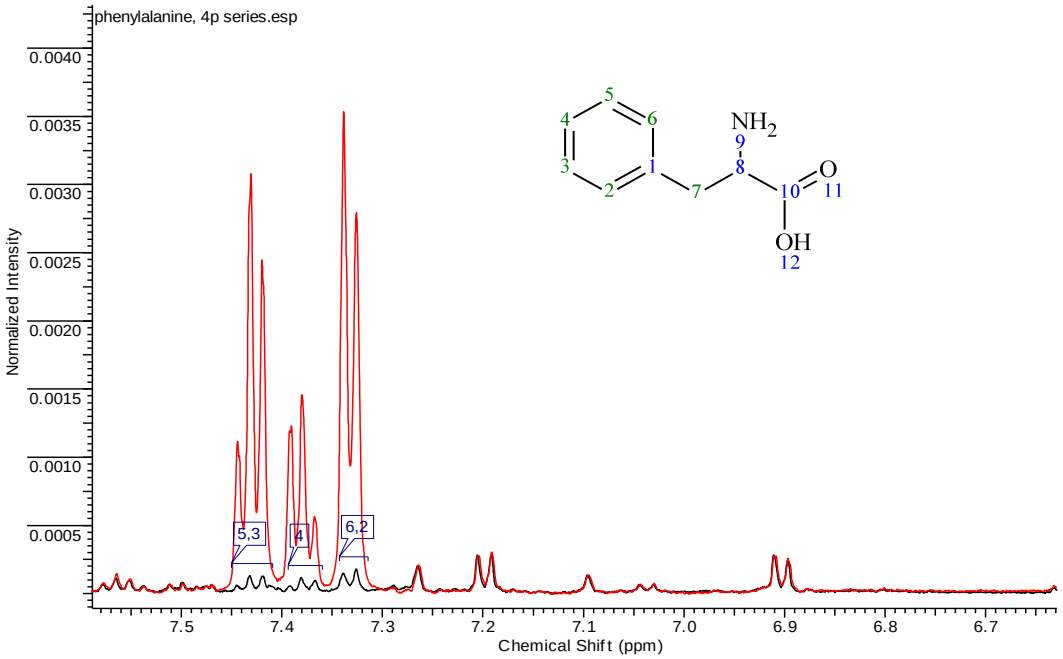


Figure 3.8. n-o. Peak positions of phenylalanine after standard addition with corresponding chemical structure. The red line is the spectrum after the addition of phenylalanine (5 μg), with the numbered boxes corresponding to the proton highlighted within the structure.

p

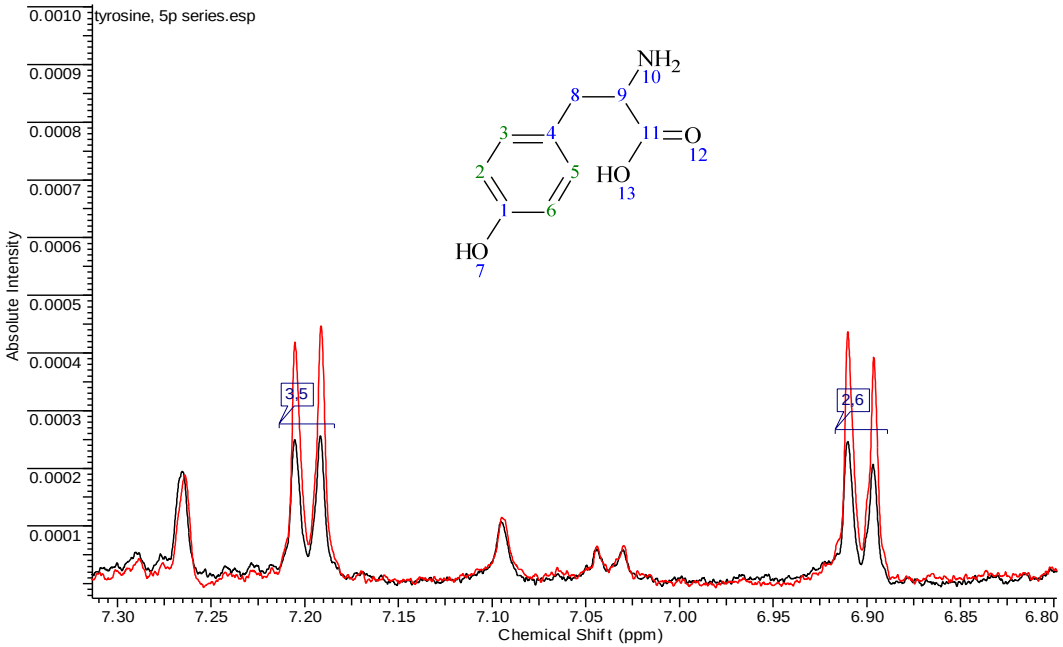


Figure 3.8. p. Peak positions of tyrosine after standard addition with corresponding chemical structure. The red line is the spectrum after the addition of tyrosine (5 μg), with the numbered boxes corresponding to the proton highlighted within the structure.

q

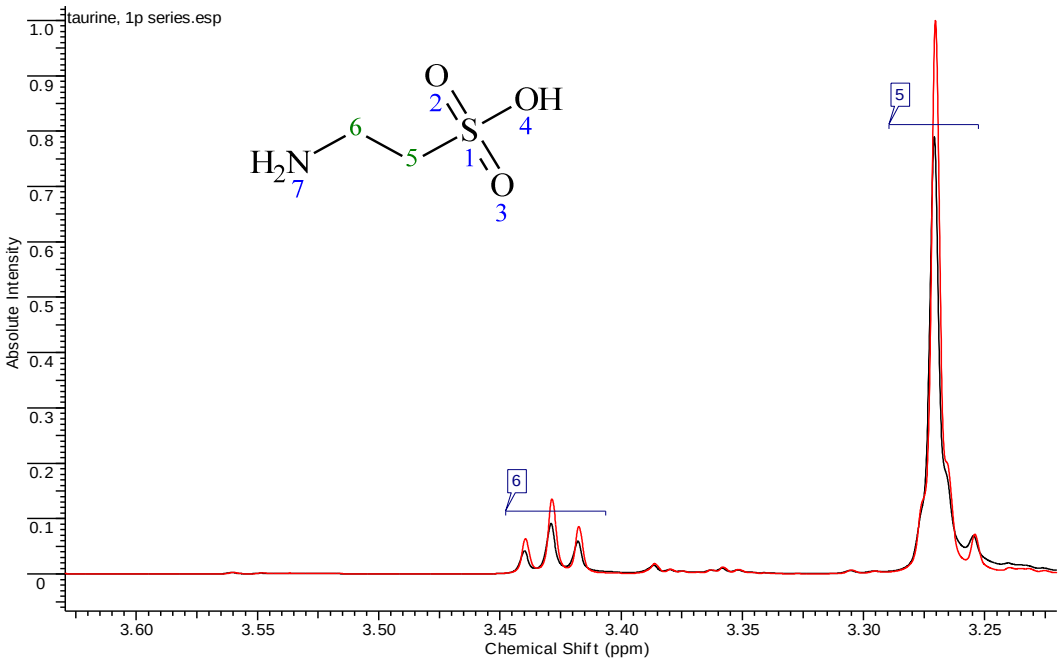


Figure 3.8. q. Peak positions of taurine after standard addition with corresponding chemical structure. The red line is the spectrum after the addition of taurine (5 μg), with the numbered boxes corresponding to the proton highlighted within the structure.

r

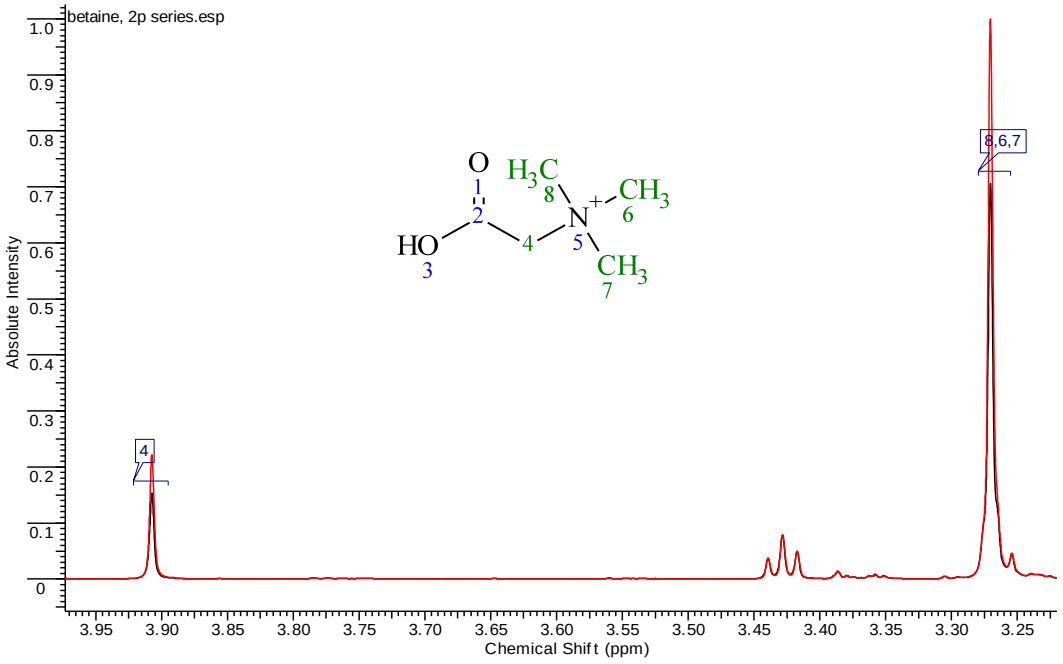
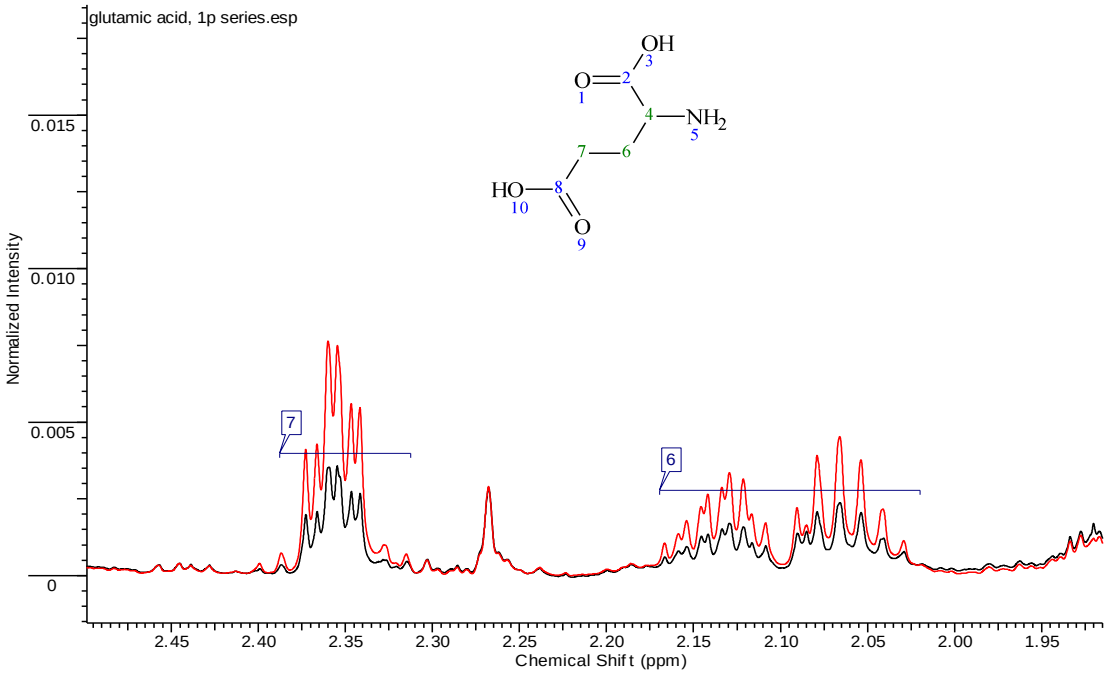


Figure 3.8. r. Peak positions of betaine after standard addition with corresponding chemical structure. The red line is the spectrum after the addition of betaine (5 μg), with the numbered boxes corresponding to the proton highlighted within the structure.

s



t

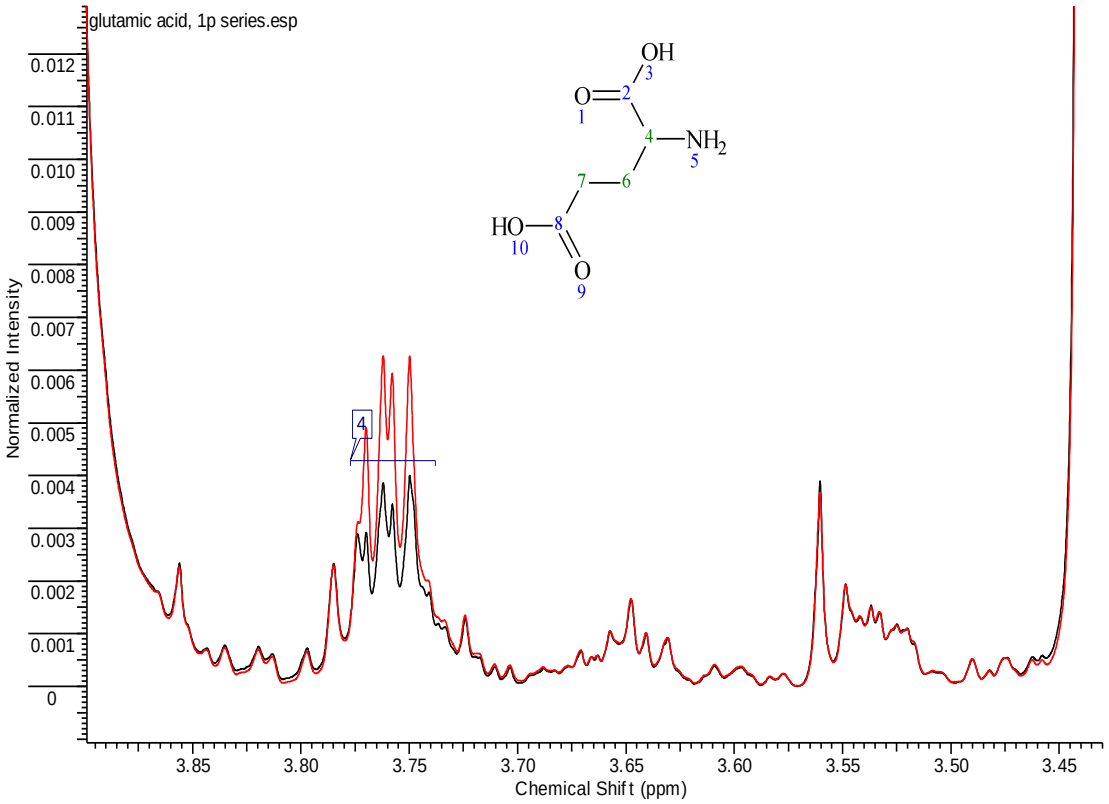
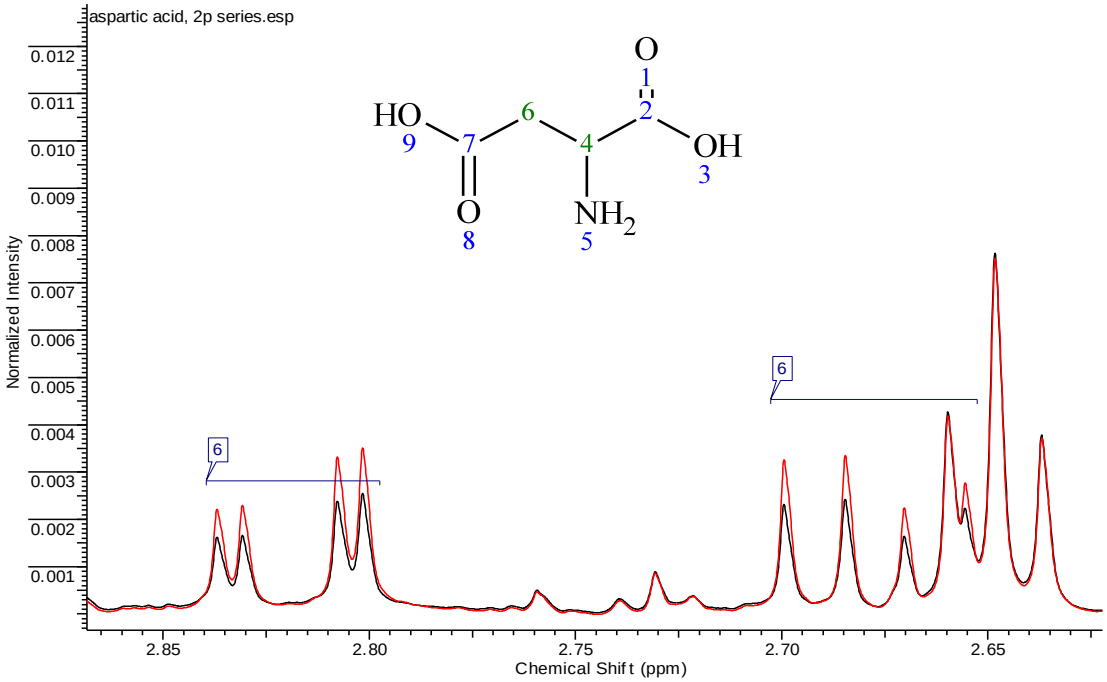


Figure 3.8. s-t. Peak positions of glutamate after standard addition with corresponding chemical structure. The red line is the spectrum after the addition of glutamate (5 μg), with the numbered boxes corresponding to the proton highlighted within the structure.

u



v

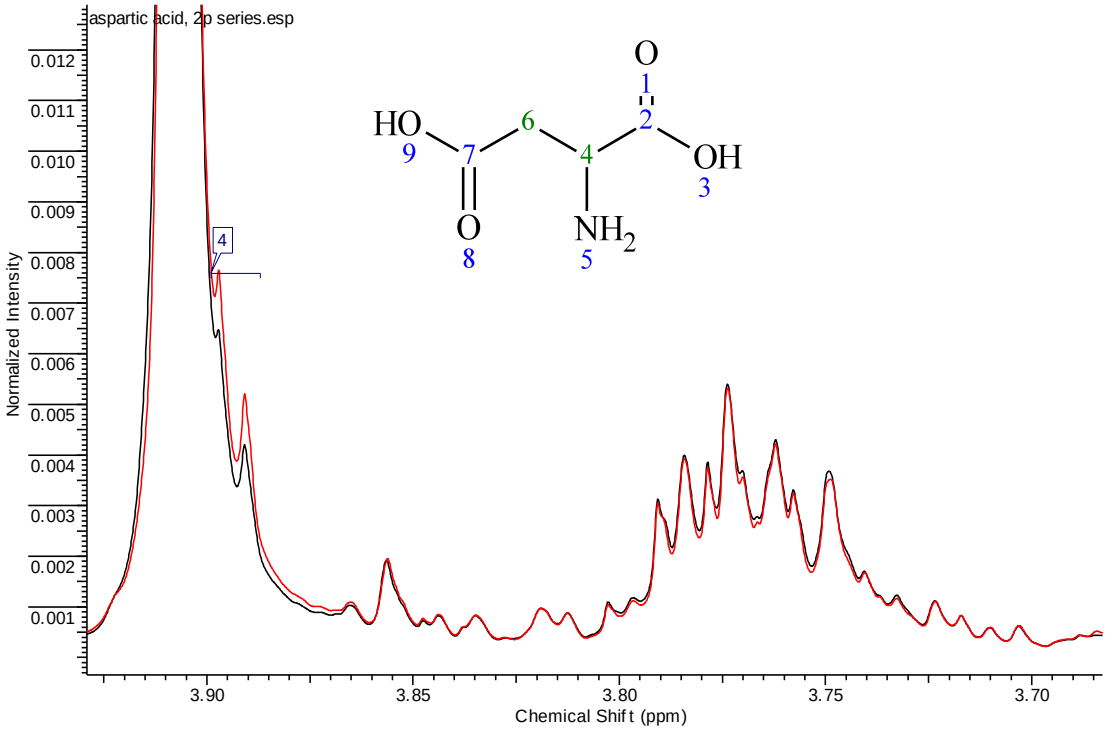


Figure 3.8. u-v. Peak positions of aspartate after standard addition with corresponding chemical structure. The red line is the spectrum after the addition of aspartate (5 μg), with the numbered boxes corresponding to the proton highlighted within the structure.

w

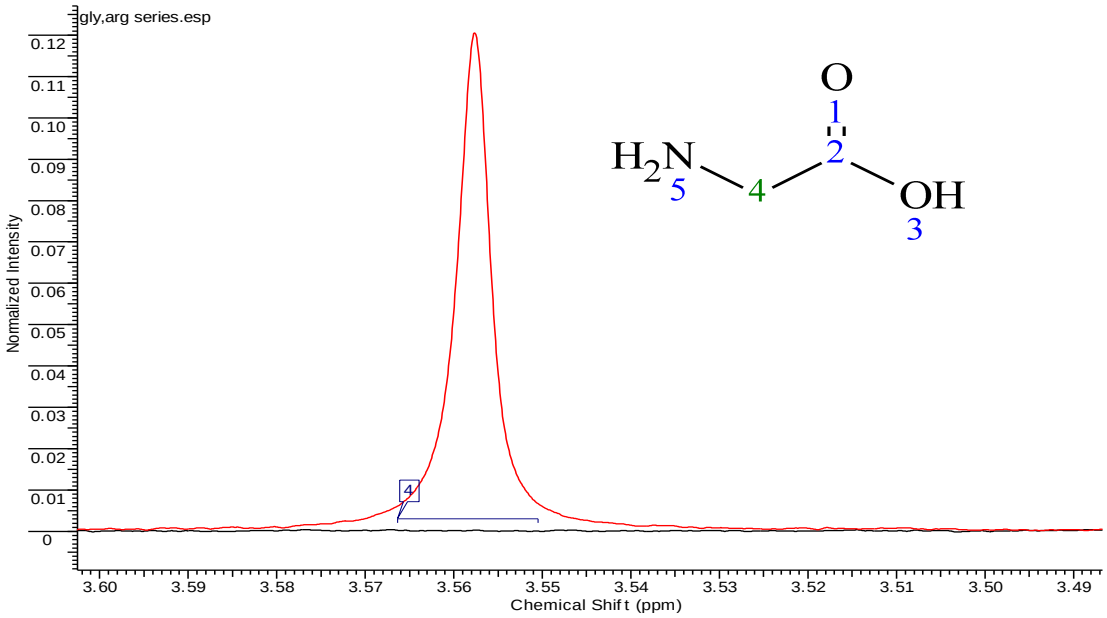


Figure 3.8. w. Peak position of glycine after standard addition with corresponding chemical structure. The red line is the spectrum after the addition of glycine (5 μg), with the numbered box corresponding to the proton highlighted within the structure.

The addition of arginine and glycine to extracts of foot tissue gave only a small increase in peaks corresponding to the protons in these compounds, making it difficult to identify them. These amino acids were therefore dissolved in an artificial sample containing only $^{2}H_2O$ for identification. The amino acid threonine displayed a doublet peak formation at ~1.33 ppm that corresponds to a similar doublet peak of lactate in the same position, therefore the other peaks at 3.58 (d) ppm and 4.27 (q) ppm were examined in order to differentiate between the two metabolites. An increase/decrease of the peak at 1.33 ppm alone without a corresponding increase/decrease at the other peak positions (3.58 (d) ppm and 4.27 (q) ppm) would indicate that lactate was the most likely metabolite.

3.5.3. Metabolic profiles of healthy control animals

During the experimental period the animals were sampled during the winter months when gametogenesis is at a minimum, therefore the gender was not identified, and profiles were from a mixture of male and female animals. This will have contributed to the background noise in the separation of control and treated individuals (Hines et al., 2007). The foot tissue was selected because it is easily identifiable, and separable from the hepatopancreas (to avoid contamination with ingested material), and gives sufficient material for extraction and metabolite analysis. This tissue provides a representative picture of the state of the metabolic pool. In other NMR studies in marine molluscs, mantle tissue, digestive gland, and haemolymph have also been used (Hines et al., 2007, Viant et al., 2003). A constant weight (100 mg) of dry tissue was extracted for use in all experiments to enable comparisons on a dry weight concentration basis. The use of acetonitrile in combination with water (Section 2.3) for the extraction of mussel foot tissue provided clean spectra (Fig. 3.9) in which numerous individual metabolites could be assigned (Table 3.1). Some of these were confirmed by spiking with chemical standards. The spectra included amino acids, carbohydrates, glycolytic products, Kreb cycle intermediates, nucleotides, organic acids, and osmolytes. The latter were the most dominant compounds in the spectrum. The range of metabolites is comparable with that found in marine molluscs by other workers (Hines et al., 2007, Viant et al., 2003).

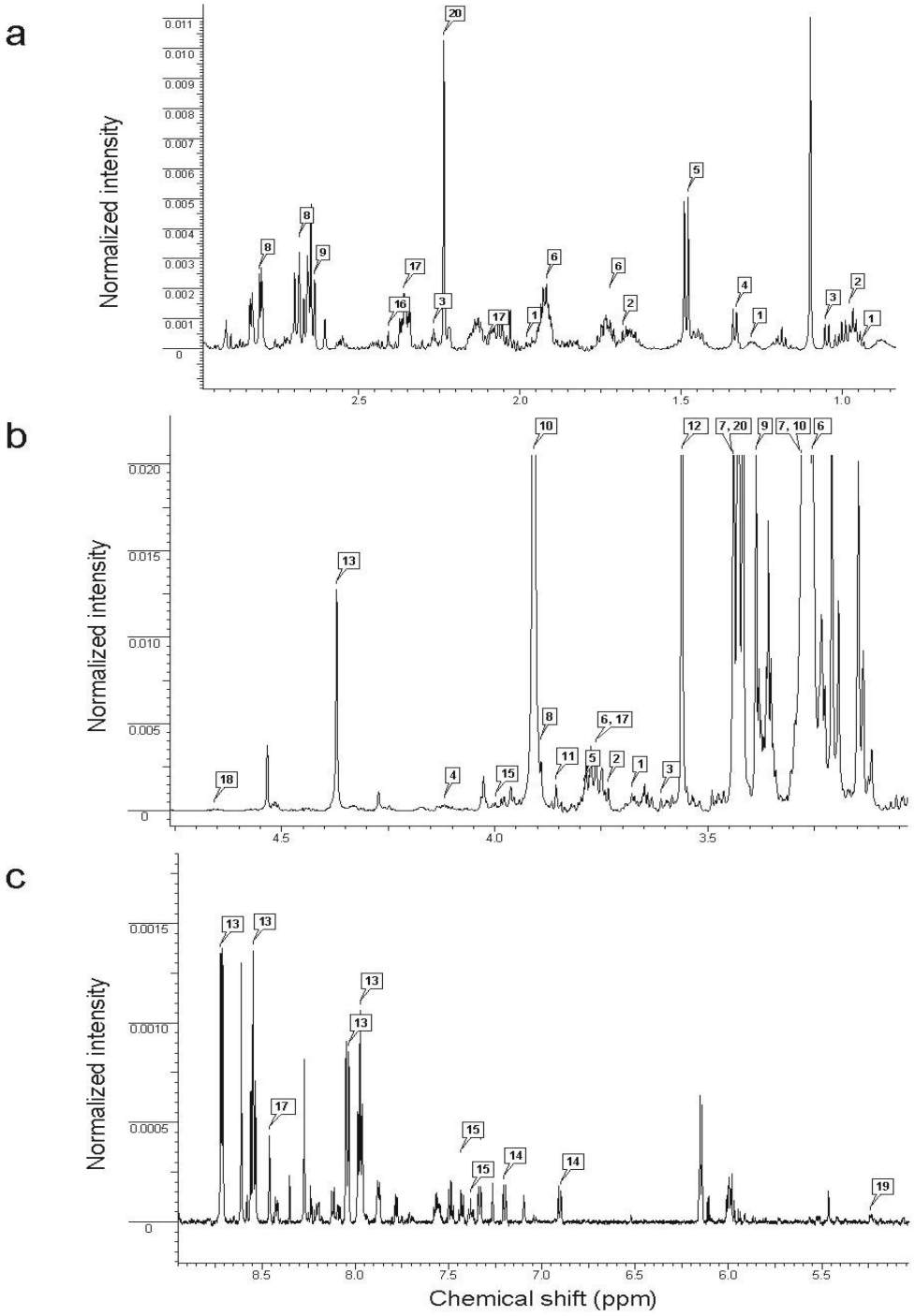


Figure 3.9. a-c. Representative one-dimensional ^{1}H NMR spectrum of the foot muscle from a healthy marine mussel (*Mytilus edulis*). The figure has been enlarged into three sections for better clarity with a and b representing the aliphatic region and c, the aromatic region with identified metabolite peaks numbered. Key to metabolites: 1. isoleucine, 2. leucine, 3. valine, 4. lactate, 5. alanine, 6. arginine/phosphoarginine, 7. taurine, 8. aspartate, 9. hypotaurine, 10. betaine, 11. serine, 12. glycine, 13. homarine, 14. tyrosine, 15. phenylalanine, 16. succinate, 17. glutamate, 18. β-glucose, 19. α-glucose, 20. acetoacetate.

Table 3.1. Metabolites present in *Mytilus edulis* foot muscle with corresponding peak values (ppm) and peak shapes: s, singlet; d, doublet; t, triplet; dd double doublet; m, multiplet.

Metabolites	Chemical shift and peak shape	Peak identification (figure 3.9)
Amino acids		
Alanine	1.48 (d), 3.78 (m)	5
Arginine/ phosphoarginine	1.70 (m), 1.92 (m), 3.25 (t), 3.76 (t)	6
Aspartate	2.68 (dd), 2.81 (dd), 3.89 (dd)	8
Glutamate	2.10 (m), 2.36 (m), 3.76 (t)	17
Isoleucine	0.94 (t), 1.02 (d), 1.28 (m), 1.98 (m), 3.68 (d)	1
Leucine	0.96 (d), 0.98 (d), 1.68 (m), 3.73 (t)	2
Phenylalanine	4.00 (m), 7.33 (d), 7.38 (t), 7.43 (m)	15
Serine	3.86 (m), 3.98 (m)	11
Tyrosine	6.90 (d), 7.21 (d)	14
Valine	1.00 (d), 1.05 (d), 2.27 (m), 3.61 (d)	3
Organic acids		
Lactate	1.33 (d), 4.12 (q)	4
Energy related		
α-Glucose	5.24 (d)	19
β-Glucose	4.66 (d)	18
Osmolytes		
Betaine	3.27 (s), 3.91 (s)	10
Glycine	3.56 (s)	12
Hypotaurine	2.66 (t), 3.38 (t)	9
Taurine	3.27 (t), 3.43 (t)	7
Kreb cycle intermediate		
Succinate	2.41 (s)	16
Micellaneous		
Acetoacetate	2.24 (s), 3.43 (m)	20
Homarine	4.37 (s), 7.97 (dd), 8.04 (d), 8.55 (dd), 8.72 (d)	13

3.5.4. Behavioural reponse of *Mytilus edulis* exposed to lindane

The typical mussel feeding behaviour under controlled conditions with the addition of acetone (1ml L^{-1}) only, used as a carrier solvent, were observed to display a full valve gape of $\geq$ 9 mm (Fig. 3.10). The valve gape was accompanied by full mantle flare from both the inhalant and exhalent valves. Foot movement and byssal thread attachment were all also observed. Those organisms exposed to the lower dose of 5 μg L^{-1} of lindane spent on average 73 % of the time open compared to the controls, spending less time open the greater the exposure time (Fig. 3.13). The gape average was much reduced to half (5 mm) that of the control group (Fig. 3.10). This reduction occurred on day one and continued throughout the exposure with the lowest gape values recorded near the end of the experimentation. There was also a reduction in foot movement, with almost none observed after 18 days. Throughout the 30 day exposure there was no significant change in water temperature (~15 ºC) or pH (~8.3) (figs. 3.11 & 3.12) and ammonia levels did not rise above the lowest limit of detection at 0.1 mg/L.

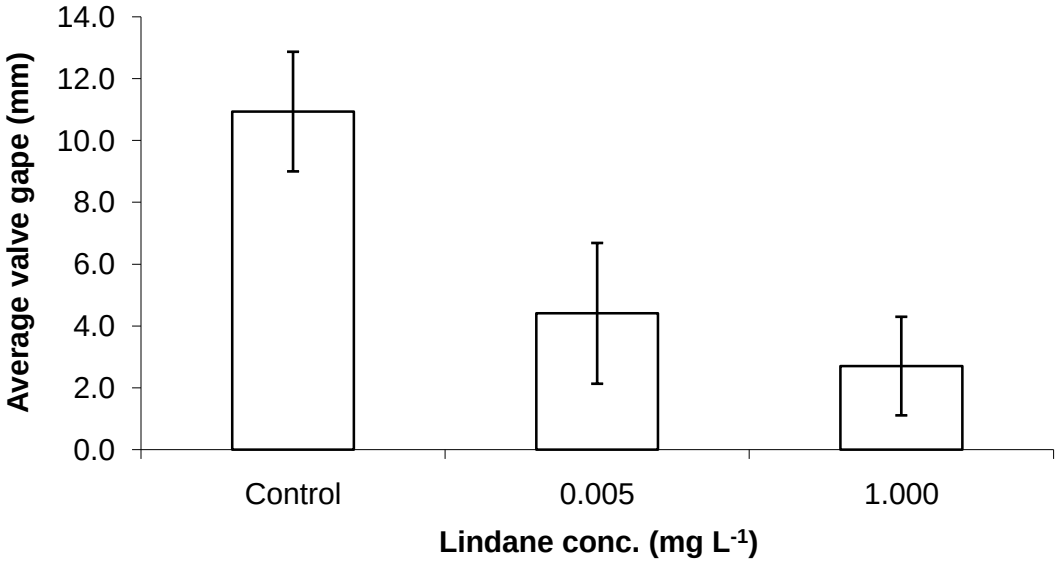


Figure 3.10. Average valve gape size (mm) between the two lindane dosed populations and that of the control group, with bars depicting the range. One way ANOVA gives p-value 0.000 and Tukey's pairwise shows that there is a significant difference between the dosed groups and that of the control.

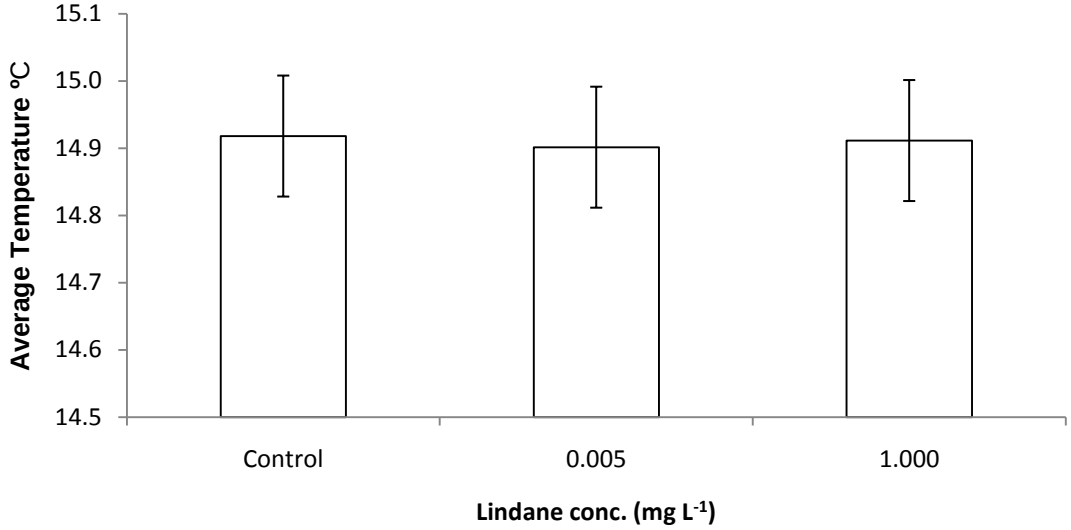


Figure 3.11. Average temperature of the test water between the two lindane dosed populations and that of the control group, with bars depicting the range. One way ANOVA gives p-value 0.945 and Tukey's pairwise shows that there is no significant difference between groups

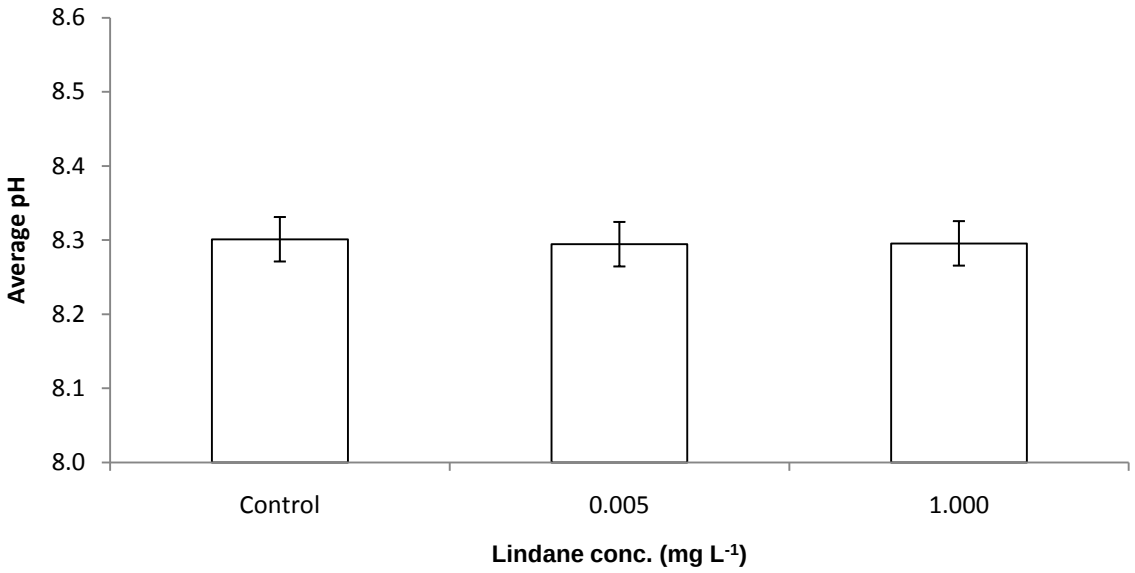


Figure 3.12. Average pH of the test water between the two lindane dosed populations and that of the control group, with bars depicting the range. One way ANOVA gives p-value 0.385 and Tukey's pairwise shows that there is no significant difference between groups

The high exposed group (1 mg L^{-1}) spent on average over the 30 day period only 43 % of the time open, where the mussels spent less and less time open and exposed to the toxicant as the experiment progressed (Fig. 3.13) Again the valve gape average was further reduced to 2.75 mm (Fig. 3.10) and almost no foot activity observed from the outset.

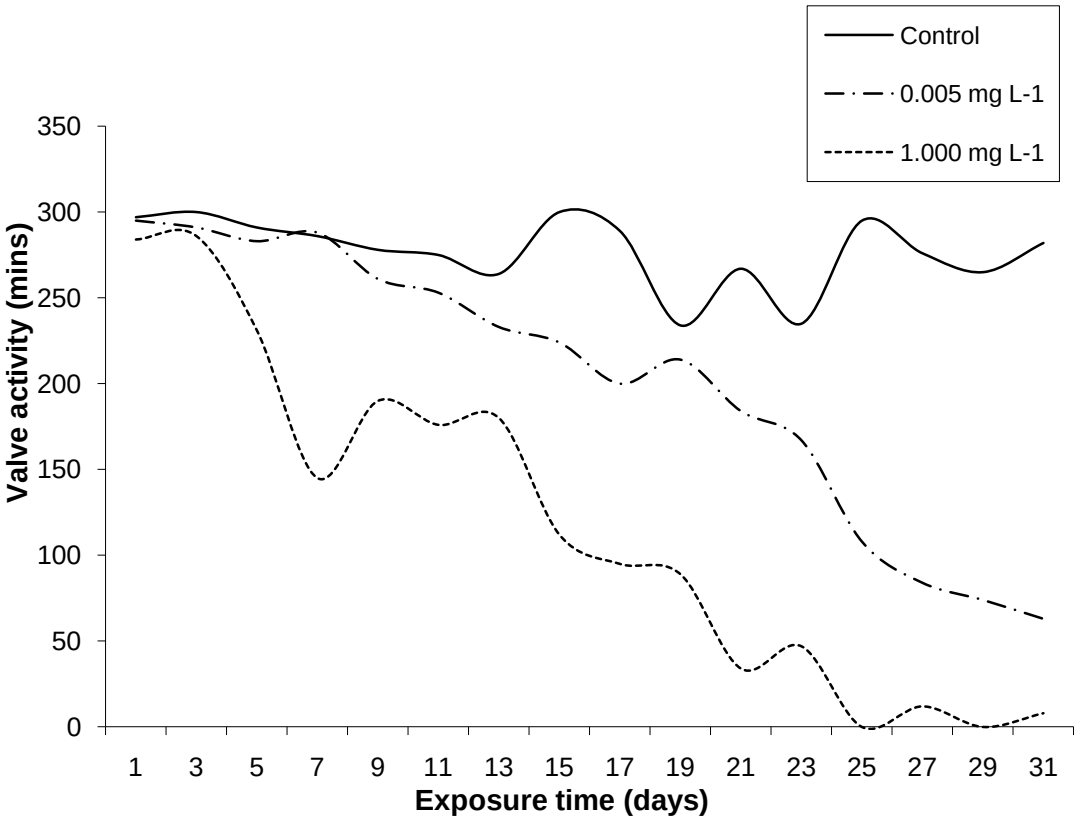


Figure 3.13. A representation of valve activity in the first 300 minute period of 24 hour renewal cycle of mussels exposed to high and low lindane concentrations compared to the control group over the 30 day exposure period.

Both groups of exposed organisms displayed an increase in oxygen saturation levels during the experiment, until the time valves spent closed significantly increased, then the oxygen saturation levels decreased, with the low dose group showing a delay of 7 days of the of the high dose group (Fig. 3.14). As the exposure time increased the mussels began to display a ‘gulping’ motion with the valves opening and closing for short bursts of time. These could be determined as a convulsive reaction to the lindane as an antagonist of the GABA receptor-chloride channel complex.

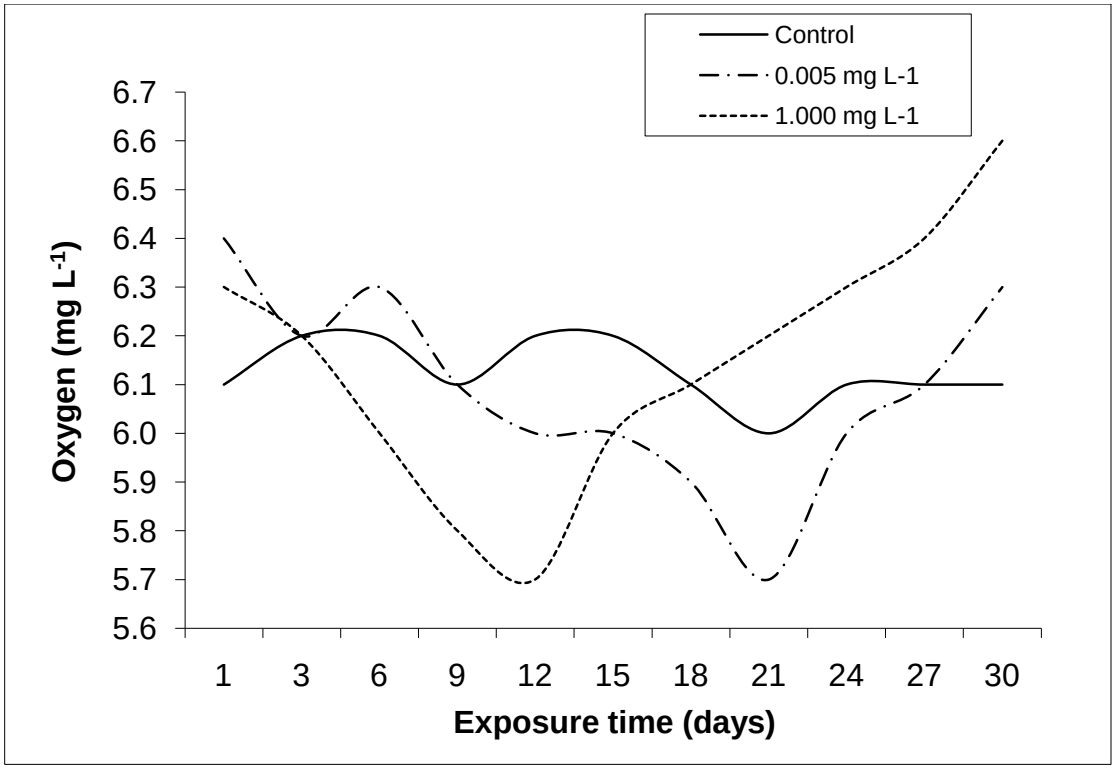


Figure 3.14. The reduction in oxygen saturation of the lindane test water after 24 h before the daily renewal recorded throughout the exposure period. The oxygen saturation at the start of each renewal was ~ 9.0 mg L^{-1}.

The reduction in valve opening of both time and gape would lead to a reduction in the organisms' ability to feed sufficiently as well as obtaining oxygen from their environment for aerobic respiration (De Zwaan and Eertman, 1996, Wang and Widdows, 1993). Therefore, over a long exposure time the scope for growth of the organism would be compromised leading to a reduction in size and overall health as well as having a significant detrimental affect on the reproduction of this semelparous species (Widdows et al., 2002).

3.5.5. Metabolic effects of exposure to lindane

The metabolic effects of the exposure were investigated by identifying the metabolites that displayed a change due to the exposure. The data from the binning process was analysed using the multivariate statistical method of PCA. The resultant three dimensional PCA plot of the original data set containing all the effects of noise within the spectra was plotted using the first three PC's (fig 3.15). There is clear separation between the three groups and the PC's were then used as descriptor variables in a discriminant analysis, and the plot of the first two discriminant functions (accounting for 89 % and 11 % of the total variation respectively). To check the quality of the supervised classification analysis (LDA) an evaluation of the (mis)classification error rate from the cross validation procedure 'leave one out' was conducted. The lindane results gave a 100 % correct group classification using all the data and 92 % after the cross validation. The robustness of the classification was further tested using a random permutation test based on 50 repetitions of the LDA using randomised indicator variable for the treatments. This gave an average correct classification of 34 % with none better than 63 %. The low correct classification rates show that the observed pattern is robust since this would be highly unlikely to occur in this pattern randomly.

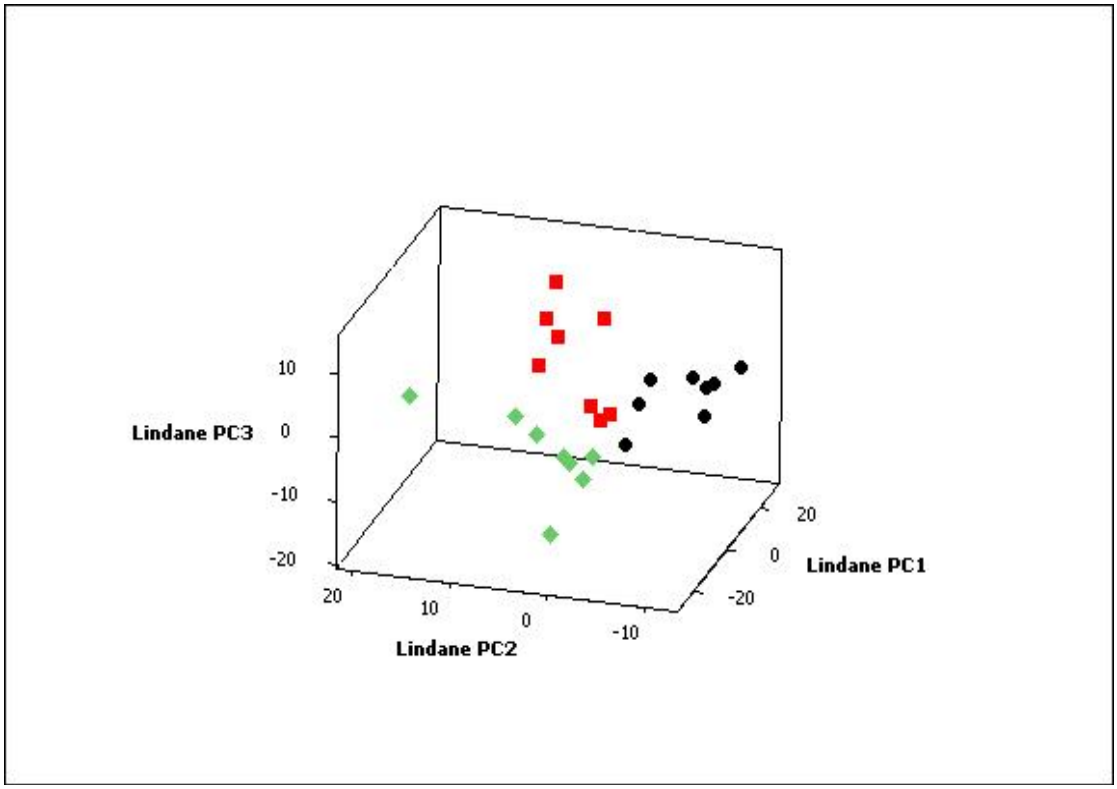


Figure 3.15. A 3-dimensional PCA scores plot showing the group variable separations of the original full lindane data set of the first three PC's. Black circles-control; red squares-low dose: green diamonds-high dose.

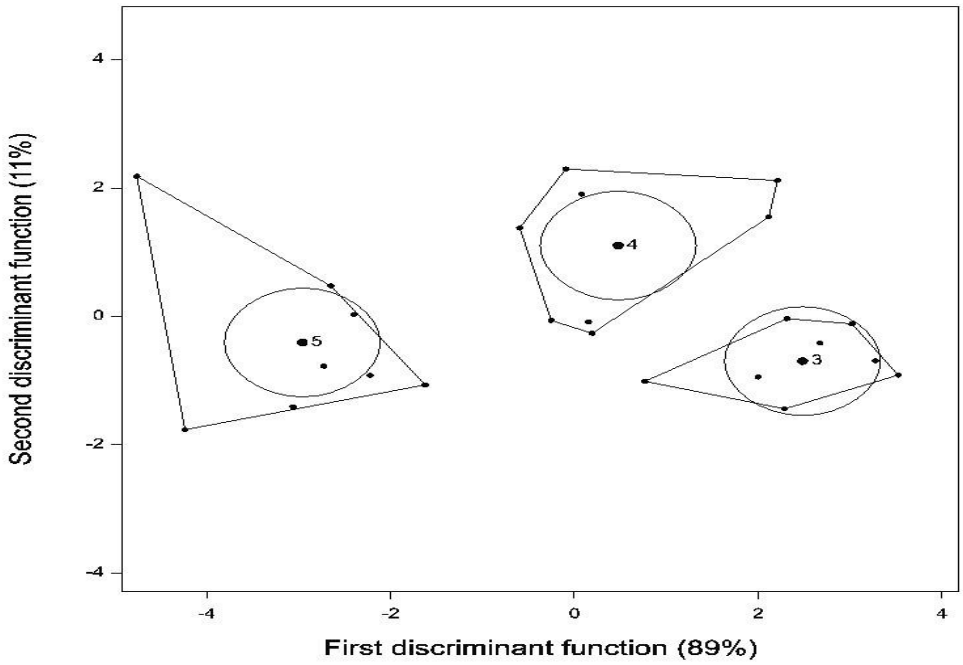


Figure 3.16. The scores on the first two discriminant functions based on the original full lindane data set. (5 - control; 4 - low dose of lindane; 3 - high dose of lindane). The positions of the treatment means are indicated by the circles.

The subsequent loadings of these PC's were used to identify those peaks (bins) associated with the highest variability, therefore reducing the effect of background noise. These new PC's where then used as descriptor variables in a LDA. he loading's from these PC's were then used An LDA was conducted on the set of variables (transformed where necessary on the basis of Box-Cox plots) that showed a statistically significant effect of treatment. The score plot (Fig. 3.17) resulting from LDA showed a clear separation of the controls, low dose (5 µg L^{-1}) of lindane, and high dose (1 mg L^{-1}) of lindane. The first discriminant function (94 % of variation) separates the controls from the lindane treated metabolic profiles. The low dose is nearer to the controls than the higher dose and this is consistent with the expected dose-response relationship. On the basis of the correlations of the original variables with the LDA axes, exposure to lindane is associated with an increase in the concentration of alanine and decreases in all of the other metabolites (the amino acid aspartate; the osmolytes betaine, homarine, taurine; the Kreb cycle intermediate succinate; and three unknowns at 1.10, 6.10 and 8.27 ppm). The second dimension accounts for only a small proportion (6 %) of the variation, and will not be interpreted here.

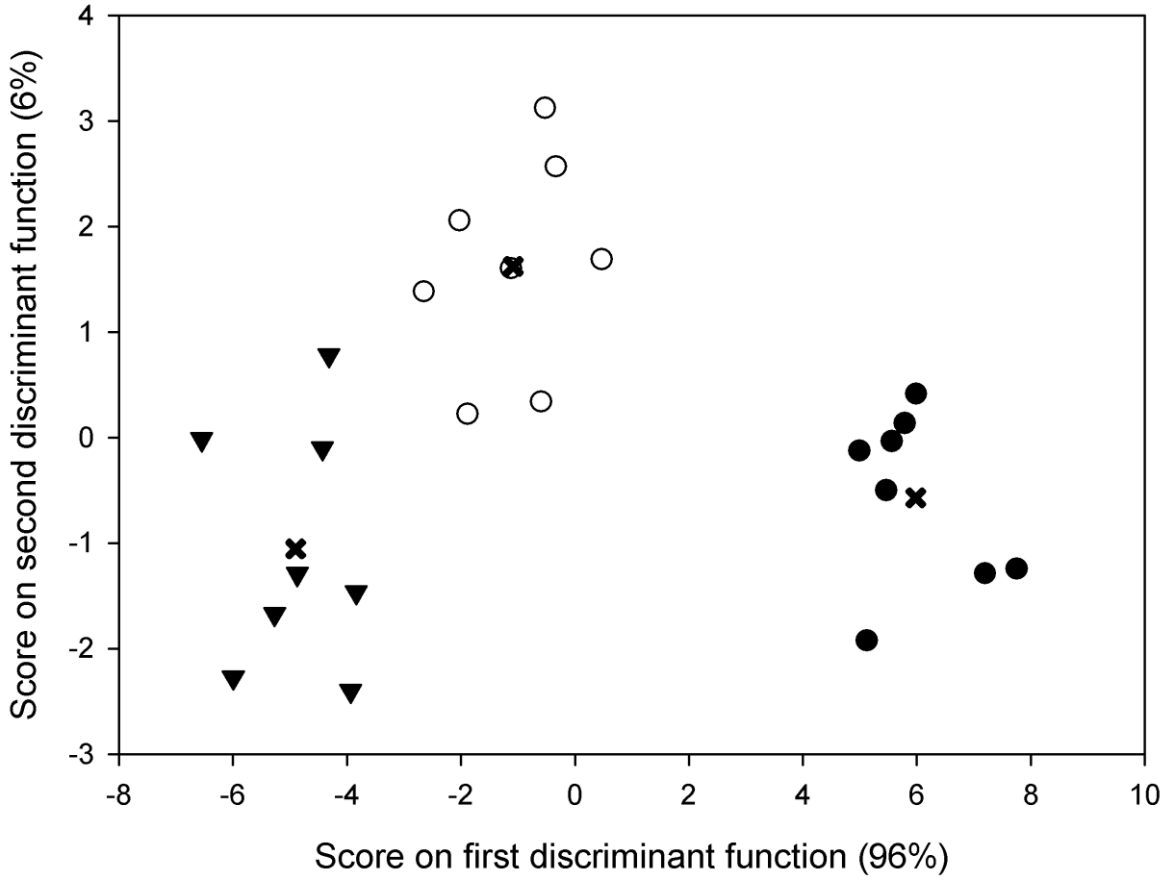


Figure 3.17. The scores on the first two discriminant functions based on the metabolites that were significantly different between the treatments (closed circles - control; open circles - low dose of lindane; closed inverted triangles - high dose of lindane). The positions of the treatment means are indicated by crosses.

The changes between these metabolites were analysed using an ANOVA and subsequent Box-cox plots. This data set was then used to identify the fold-change of the metabolite concentration (peak area) from that of the control and in which direction (increase/decrease) with the results in table 3.2. To report on the quality of the supervised classification analysis (LDA) a permutation test was conducted to analyse the (mis)classification error rate from the cross validation procedure 'leave one out'. The original data set gave a 100 % correct variable grouping, the 'leave one out' procedure lead to the

misclassification of two samples were low dose lindane samples were classified as controls. The permutation test was conducted 50 times where the average correct group classification was 34 % and 68 % were below 33 % with only three sets going as high as 63 % correct. This shows that although with the 'leave one out' analysis two samples were misclassified this still gave a correct classification of 88 % much higher than those that may occur by chance.

Table 3.2. Table of metabolic changes of low and high dose lindane exposure showing the fold-change from the control and p-value from the ANOVA. The arrows indicate whether an increase (↑) or decrease (↓) was identified.

Metabolite	Low dose lindane Fold change	High dose lindane Fold change	p-value
Alanine	↑ 1.8	-	0.069
Aspartate	↓ 1.7	↓ 4.8	0.000
Betaine	-	-	0.026
Homarine	-	-	0.028
Succinate	↑ 2.5	-	0.057
Taurine	-	-	0.047
Ukn (1.10 ppm)	-	↓ 1.6	0.035
Ukn (6.10 ppm)	-	↓ 1.9	0.018

Animals treated by El Shenawy *et al*. (2006) with the higher concentration (0.935 mg L^{-1}) of lindane were observed after 28 days of exposure to exhibit a five-fold increase in ammonia production compared with controls. This was associated with a six-fold decrease in energy consumption, and a ten-fold decrease in energy absorption. This was consistent with a five-fold decrease in feeding rate, a sixty-fold decrease in the ratio of valve open to valve closed time, and a reduced gape. In that study there was a twelve-fold increase in the oxygen to nitrogen ratio indicating that the treated animals were utilising more

protein relative to carbohydrates and lipids than the controls (El-Shenawy, 1999). This combination of symptoms indicates that the treated animals are starving. These animals were also showing reduced ventilation rates and so the effects of starvation may have been combined with those of reduced oxygen supply.

An increase in levels of alanine have been observed in a range of invertebrate species (*Deroceras species* (Storey *et al.*, 2007), *Crassostrea gigas* (Michaelidis *et al*., 2005), crayfish (Abe, 2002), *Patella caerulea* (Santini *et al.*, 2001), *M. galloprovincialis* (Bacchiocchi and Principato, 2000, Isani *et al.*, 1995), *Macoma balthica* (Ahmad and Chaplin, 1984), and *Limulus polyphemus* (Carlsson and Gade, 1986) when subjected to hypoxic conditions for different periods of time. In some of these studies the increase in alanine caused by anoxia was associated with an increase in succinate. Accumulation of succinate is a clear indication of facultative anaerobiosis in molluscs (de Zwaan and Wijsman, 1976). This was not observed in the lindane poisoned mussels in the current study where the increase in alanine concentration was accompanied by a decrease in the other analytes (including succinate) that contributed to the discrimination. These differences may be attributable to wide range of exposure regimes used in the various studies. Alanine is an important osmolyte in many invertebrates (Abe *et al*., 2005), and is found in high concentrations, as well as being produced in anaerobic metabolism (Carlsson and Gade, 1986). The mussels in the current study were exposed to lindane over a 28 day period, and at the end of this at the higher dose were shown by (El-Shenawy, 1999) to have negative scope for growth. This is

consistent with the general decrease in metabolites from a range of classes observed in this metabolomic study. It is difficult to associate this with the known specific mode of action of lindane in insects, and the observed symptoms may be associated with non-specific toxicity caused by accumulation of this non-polar compound in the body lipids of the mussel, and the prolonged decrease in feeding efficiency and ventilation rate.

3.5.6. Behavioural response of *Mytilus edulis* exposed to atrazine

The exposure of the test species to atrazine displayed a behavioural response similar in some aspects to that of the lindane exposed organisms where valve gape, the size of the distance between valves during respiration and feeding, was reduced from the start of the exposure to < 6 mm at the low dose and < 4 mm at the higher dose level compared to an average gape size of ~11 mm observed in the controls (Fig. 3.18). The valve activity of the mussels also reduced over time, but not to the extent of that of the lindane exposed organisms, with the low dose spending an average of 83 % of the control average opening time and the high dose at 60 % (Fig .3.19). In contrast to lindane as valve activity decreased there was a marked increase in apparent oxygen consumption after 20 days exposure, as the oxygen saturation levels at the end of each 24 h period were much reduced (Fig. 3.20). This follows the pattern investigated in a previous study where the oxygen level was measured using ‘closed’ glass respirometers (El-Shenawy et al., 2006). This coincided with a behavioural change, as the water was removed from the test vessels

instead of the mussel valves closing they remained open whilst exposed to the air. This could be the result of the increase in oxygen consumption with the mussels extracting oxygen from the air. The valves would eventually close with a little physical stimulation (tapping on the shells) that would suggest the open state was voluntary. Throughout the 30 day exposure there was no significant change in water temperature (~15 ºC) or pH (~8.2) (figs. 3.21 & 3.22) and ammonia levels did not rise above the lowest limit of detection at 0.1 mg/L.

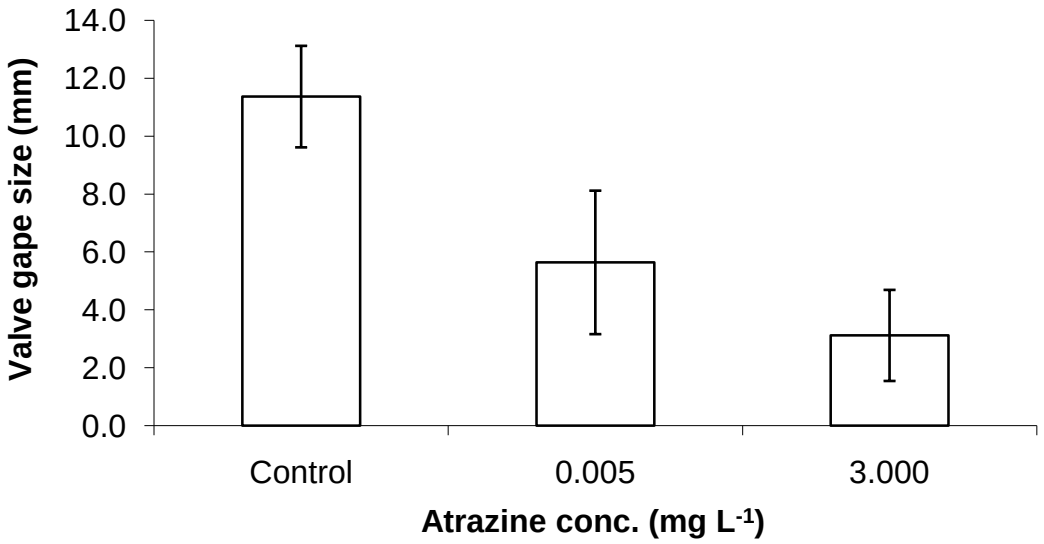


Figure 3.18. Average valve gape size (mm) between the two atrazine dosed populations and that of the control group, with bars depicting the range. One way ANOVA gives p-value 0.000 and Tukey's pairwise shows that the low and high dosed individuals gape is significantly lower than the control.

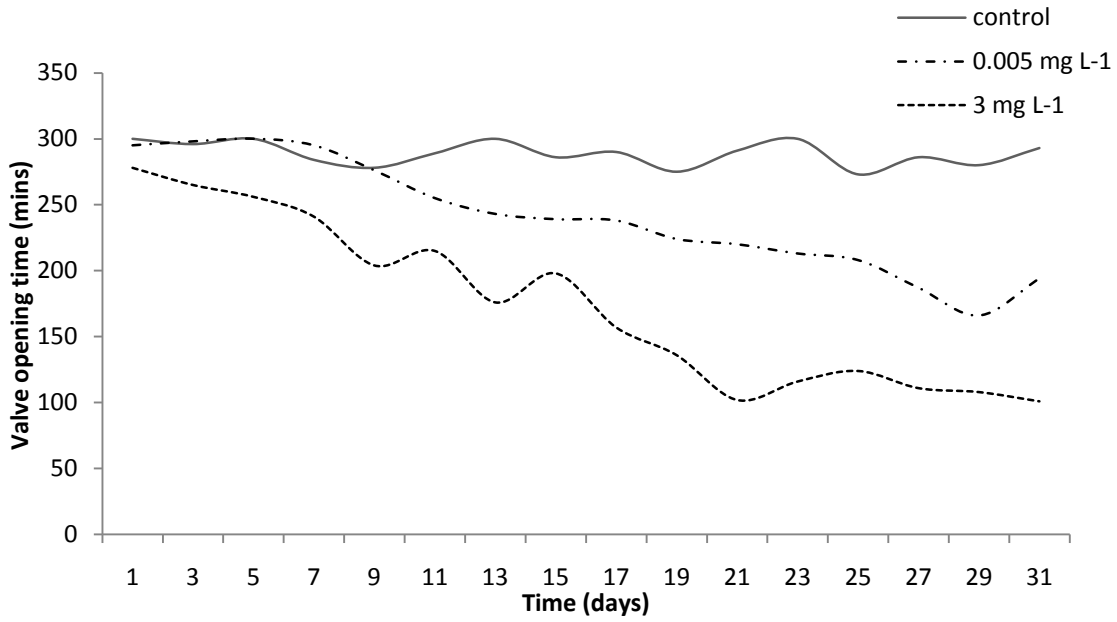


Figure 3.19. A representative valve activity in the first 300 minutes of the 24 hour renwal cycle of mussels exposed to high and low atrazine concentration compared to the control group over the 30 day exposure period.

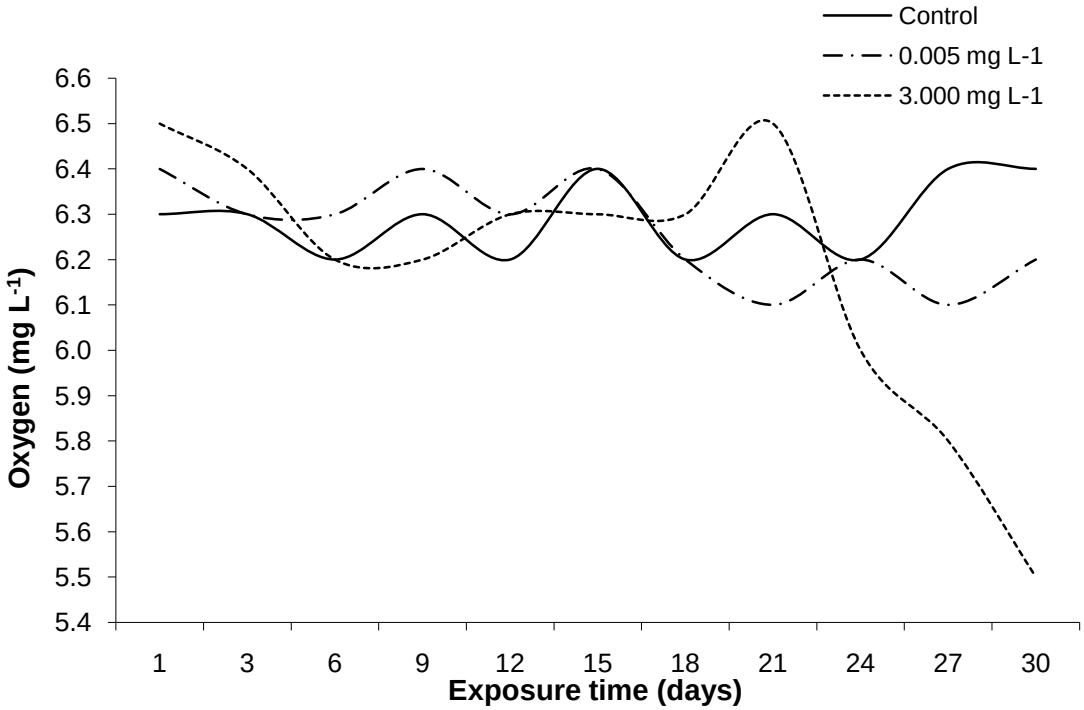


Figure 3.20. The reduction in oxygen saturation of the atrazine test water after 24 h before the daily renewal recorded throughout the exposure period. The oxygen content at the start of each renewal was ~ 9.0 mg L^{-1}.

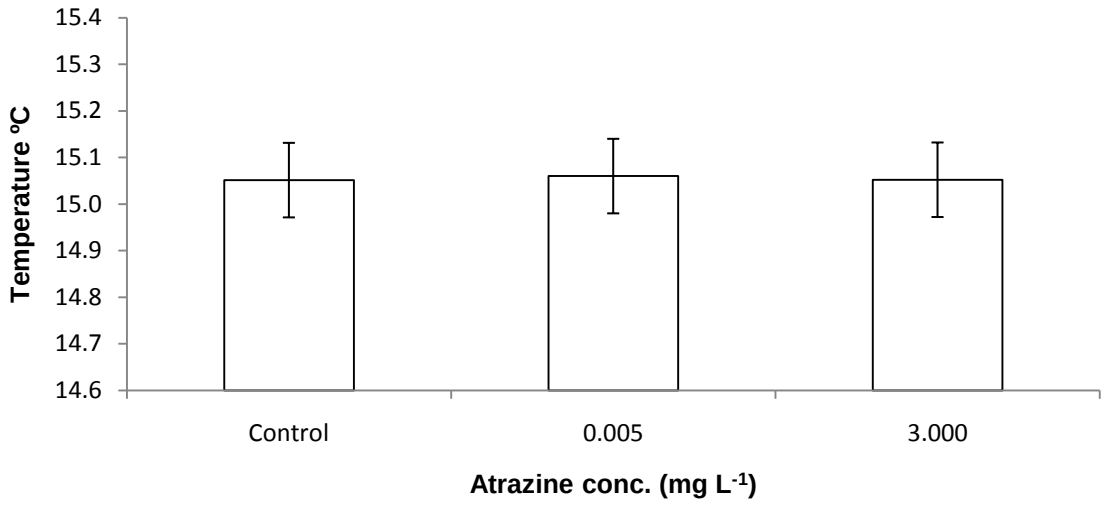


Figure 3.21. Average temperature of the test water between the two atrazine dosed populations and that of the control group, with bars depicting the range. One way ANOVA gives p-value 0.756 and Tukey's pairwise shows that there is no significant difference between groups.

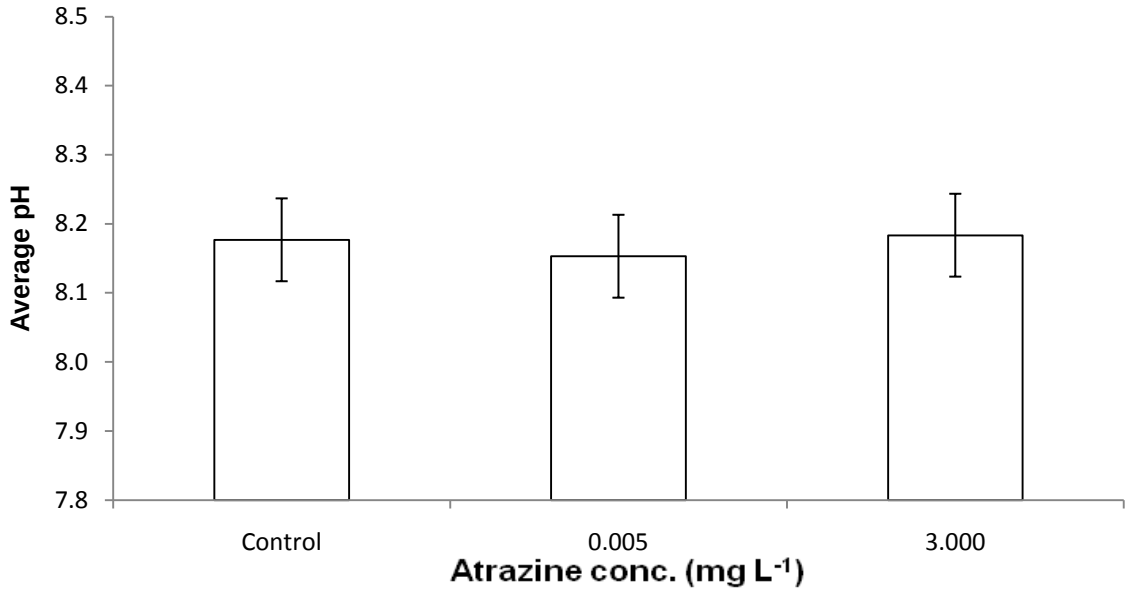


Figure 3.22. Average pH of the test water between the two atrazine dosed populations and that of the control group, with bars depicting the range. One way ANOVA gives p-value 0.553 and Tukey's pairwise shows that there is no significant difference between groups.

3.5.7. The metabolic effects of hypoxia, starvation and exposure to atrazine

The changes to the metabolic pool of the mussels due to the chemical and environmental stressors were investigated. Significant changes to metabolism were identified using PCA. A three dimensional PCA plot of the first three PC's of the original full data set containing all noise effects, with only the water peak removed is shown in figure 3.23. Due to the added effects of noise the separation is not clear. The PC's were then used as descriptor variables in a discriminant analysis, and the plot of the first two discriminant functions (accounting for 77 % and 17 % of the total variation respectively) shows overlapping of the control and the atrazine low dose exposure (fig. 3.24). The high dose group also has a single individual not distinguished from the low dose group. The quality of the supervised classification analysis (LDA) was evaluated as the (mis)classification error rate from the cross validation procedure 'leave one out'. The atrazine, starvation and hypoxia results gave a 90 % correct group classification using all the data and 78 % after the cross validation. The robustness of the classification was further tested using a random permutation tests based on 50 repetitions of the LDA using randomised indicator variable for the treatments. This gave an average correct classification of 21 % with none better than 48 %. These low correct classification rates show the robustness of the observed pattern since this would be highly unlikely to occur in this pattern randomly.

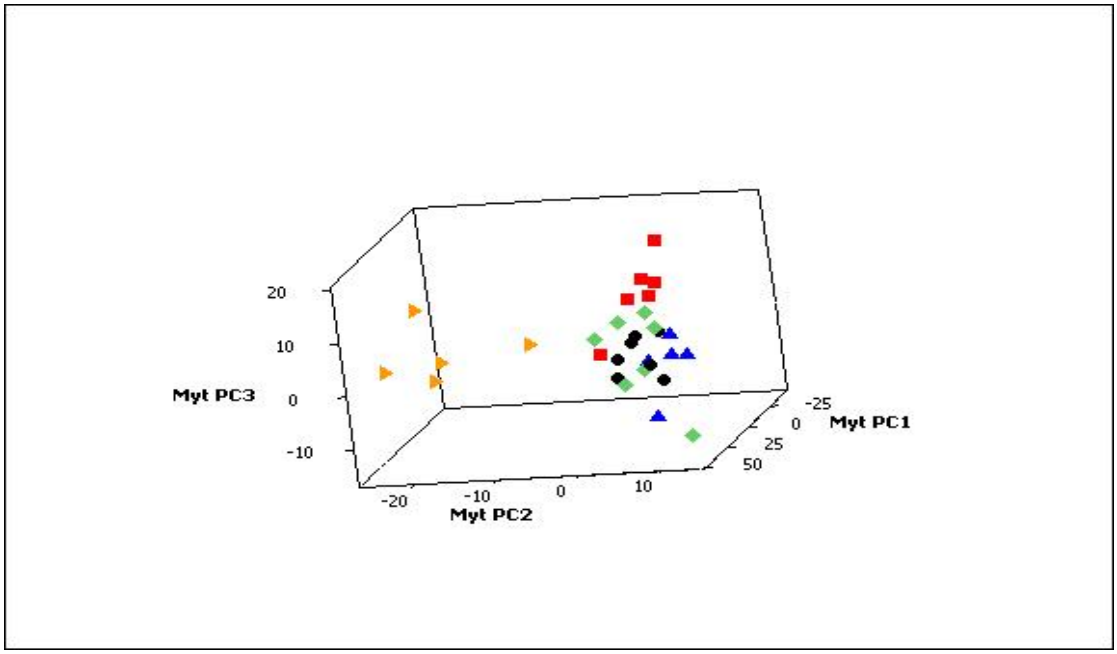


Figure 3.23. A 3-dimensional PCA scores plot showing the group variable separations of the original full atrazine data set of the first three PC's. Black circles-control; red squares-low dose: green diamonds-high dose; blue triangles-hypoxia; yellow triangles-starvation.

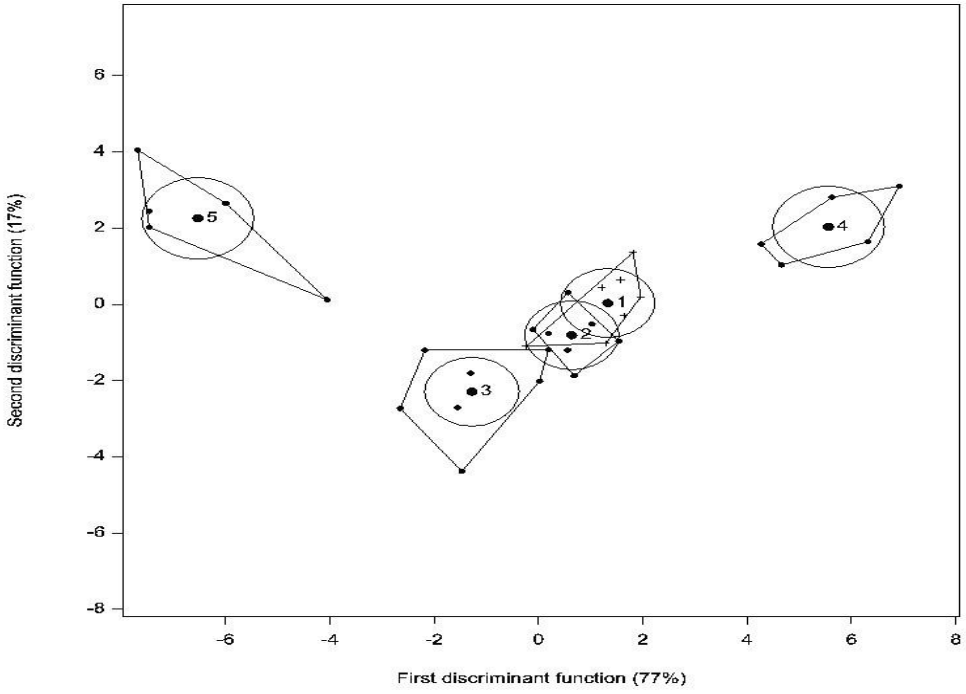


Figure 3.24. The scores on the first two discriminant functions based on the original full atrazine data set. (1 - control; 2 - low dose; 3 - high dose; 4 – hypoxia; 5 - starvation). The positions of the treatment means are indicated by the black closed circles.

From the subsequent loadings of these PC's further analysis was conducted on those peaks (bins) associated with the highest variability, therefore reducing the effect of background noise. These new PC's where then used as descriptor variables in a LDA. Here the first discriminant function (62 % of the variation) separates starvation and hypoxia from the controls, and low and high doses of atrazine from each other and from the controls and the starved animals (Fig. 3.25). The second function (25 % of the variation) separates hypoxia from the controls, and starvation and high and low doses of atrazine. The last three fall in a similar position on this axis. The controls fall between the low and high doses of atrazine. The original variables with the highest correlations with the scores on the second discriminant function indicate that hypoxia (relative to controls) is associated with increased levels of succinate and valine, and decreased levels of isoleucine, leucine, and a number of unidentified peaks (at 4.02, 6.10 and 8.24, 8.27 and 8.61 ppm). For atrazine treatment and starvation that fall above the controls on this axis, the opposite applies. Correlations of the original variables with the scores on the first discriminant function indicate that the high dose of atrazine (relative to the low dose, hypoxia and starvation) is associated with increased levels of isoleucine, leucine, and a number of unidentified peaks (at 4.02, 4.58, 8.27 and 8.61 ppm). Starvation and hypoxia relative to the controls are associated in this dimension with increased aspartate and some unknowns (at 6.10 and 8.24 ppm).

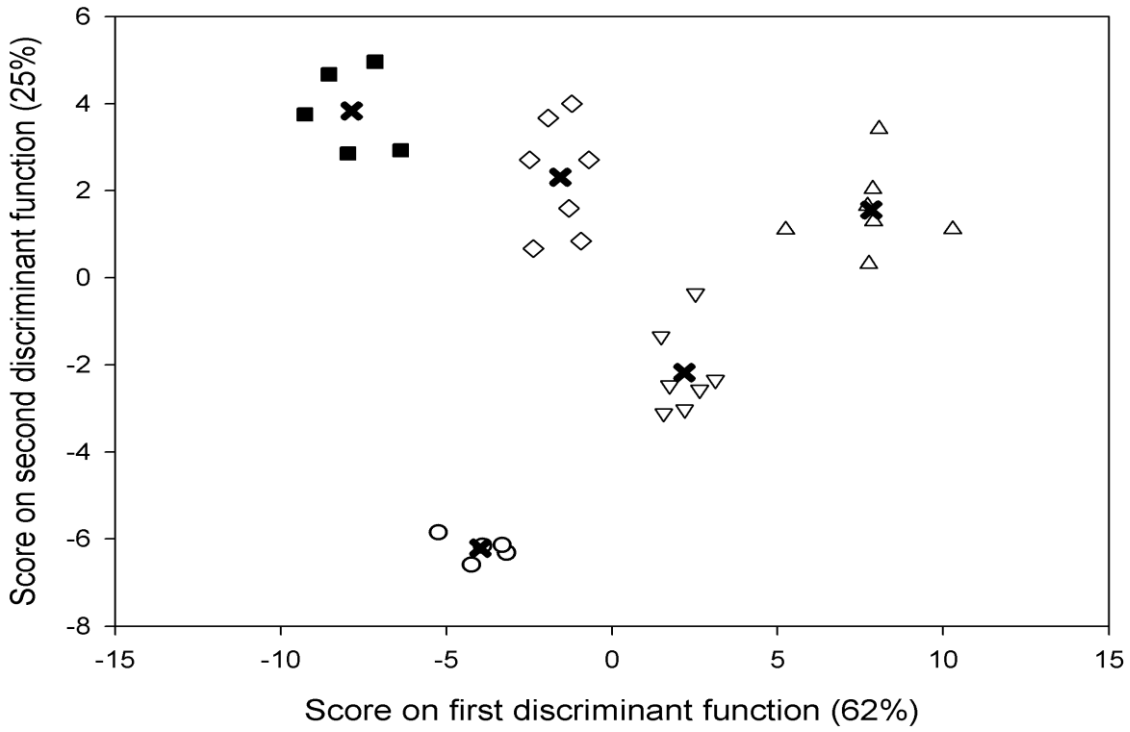


Figure 3.25. The scores on the first two discriminant functions based on the metabolites that were significantly different between the treatments (open circles - hypoxia; open inverted triangles - control; closed squares - starvation; open diamonds - low dose atrazine; open triangles - high dose atrazine). The crosses indicate the treatment means.

The changes between these metabolites were analysed using an ANOVA and subsequent Box-cox plots as with the lindane data. The data set was again used to identify the fold-change of the metabolite concentration (peak area) from that of the control and in which direction (increase/decrease) with the results in table 3.3. The quality of the supervised classification analysis (LDA) was again conducted. The atrazine, starvation and hypoxia results gave a 100 % correct group classification using all the data and after the cross validation. Permutation tests based on 50 repetitions gave an average correct classification of 20 % with none better than 33 %. These low correct classification rates show the robustness of the observed pattern since this would be highly unlikely to occur in this pattern randomly.

Table 3.3. Table of metabolic changes of low and high dose of atarazine, starvation and hypoxia showing the fold-change from the control and p-value from the ANOVA. The arrows indicate whether an increase (↑) or decrease (↓) was identified.

Metabolite	Hypoxia Fold change	Starvation Fold change	Low dose Fold change	High dose Fold change	p-value
Aspartate	↑ 1.3	↑ 1.6	↑ 1.7	↓ 1.3	0.000
Isoleucine	↑ 1.4	↑ 2.5	-	↑ 2.3	0.000
Leucine	-	↑ 2.2	-	↑ 1.6	0.000
Succinate	↑ 2.2	↓ 4.1	-	-	0.000
Valine	↓ 1.6	↑ 1.6	↓ 1.5	↓ 1.2	0.000
Ukn (4.02 ppm)	↓ 1.8	-	-	-	0.000
Ukn (4.44 ppm)	↑ 54.3	↑ 2.7	-	-	0.000
Ukn (4.58 ppm)	↑ 4.5	-	-	-	0.000
Ukn (6.10 ppm)	↑ 1.4	↑ 6.3	↑ 2.2	-	0.000
Ukn (8.24 ppm)	↑ 2.3	↑ 6.9	-	↓ 2.3	0.000
Ukn (8.35 ppm)	↓ 2.5	↑ 5.2	-	↑ 1.6	0.000
Ukn (8.58 ppm)	-	↑ 2.1	-	↑ 1.8	0.016
Ukn (8.61 ppm)	-	-	-	↑ 1.6	0.018

In an earlier investigation (El Shenawy *et al*., 2006) of the impact of a high dose (3.585 mg L^{-1}) of atrazine on the scope for growth of *M. edulis* a range of physiological and biochemical symptoms were observed after a 28 day exposure. These included a twenty-four-fold increase in ammonia production compared with controls, a halving of energy consumption, and a three-fold decrease in energy absorption. This was consistent with a sixty-fold decrease in the ratio of valve open to valve closed time, and a reduced gape. Overall a twenty-fold decrease in the oxygen to nitrogen ratio was observed (El-Shenawy, 1999). Again this syndrome is indicative of starvation. However, there was a marked contrast between the effects of this herbicide and lindane. The former had a far greater impact on scope for growth than the latter, and this was associated with an increase (four-fold) in total maintenance energy

and a decrease in energy absorption. In contrast in lindane treated mussels the total maintenance energy was similar to that of controls but the energy absorption was much lower.

In the current study the effects of hypoxia and starvation operated in different directions relative to the control. Hypoxia was associated with an increase in succinate and valine concentrations, and a decrease in leucine and isoleucine concentrations. In these experiments the expected association with increased levels of alanine was not evident in the discrimination function. The effect of atrazine relative to the controls was in the direction of starvation. The animals poisoned with atrazine were subjected to a combination of starvation and reduced ventillatory activity, as well as to the biochemical lesions produced by atrazine (El-Shenawy *et al*., 2003, 2006). Insight into the specific toxic response to the herbicide can be gained from the first discriminant function that separates the high and low doses. The higher dose is associated with increases in leucine and isoleucine concentrations. This is consistent with the demonstrated (Ciummo, 2006, El-Shenawy et al., 2006) stimulation of metabolic activity leading to an unnecessary high output of energy. (Ciummo, 2006) found this to be associated with increased citric acid cycle oxidation of succinate in fresh water mussels exposed to concentrations (1.5 to 150 µg L^{-1}) of atrazine over a period of 7 days. (McCarthy and Fuiman, 2008) found a large increase in both protein catabolism and anabolism in larvae of the fish *Sciaenops ocellatus* exposed to atrazine (40 and 80 µg L^{-1}) and this was consistent with the doubling of metabolic rate observed by (Alvarez and Fuiman, 2005) in these larvae exposed to the same dose regime. Information

on the chronic toxicity of atrazine in invertebrates is sparse. A recent GCxGC/TOF-MS metabolomic study (Ralston-Hooper et al., 2008) in an amphipod crustacean exposed to atrazine (from 0.03 to 3000 µg L^{-1}) showed that L-aspartic acid was the only significant down regulated metabolite. The highest dose was similar to that used in the current study, and the observation is consistent with the association of the scores of the high atrazine group with decreased aspartate.

In this study we investigated the toxic effects of two contrasting pesticides; a herbicide and an insecticide that would be expected to have different modes of action. The method produced a clear separation between the treatment groups, and controls, and identified some key metabolites that were associated with the effects of the toxicants. The utility of this approach to contribute to the identification of potential markers of exposure of organisms to pollutants has been demonstrated. However, interpretation of the metabolomic data is not straightforward, and is complicated by the interactions between natural environmental stressors and toxic insults due to anthropogenic pollutants. A clearer picture may be possible if the contribution of a range of biological factors including sex, age, and seasonal effects, and previous history of exposure to pollutants and environmental stressors could be removed (Viant, 2007a). There is a need for further studies using this approach to provide statistically sound measurements of the normal metabolic operating range (Viant, 2007a) of this important model species taking into account the sources of variation described above.

3.3.8. Linear dicriminant analysis of lindane and atrazine data

The standardised spectral data from both the lindane (24 samples by 671 bins) and atrazine (31 samples by 658 bins) experiments were reduced by extracting the principal components (PC's), and using linear discrimination analysis (LDA) to identify those PC's that were important in separating the treatment groups. Only the first few (larger) PC's were used in this analysis. The LDA plots for the two data sets are presented in Figs. 3.26 and 3.27 below. The treatment groups are separated in the first two dimensions. Following this procedure the metabolites associated with the PC's that contributed to the separation of groups were identified, and the original data were then transformed using Box-Cox analysis where necessary to render them normal. These were then analysed using analysis of variance and those showing a significant difference between controls and treatments were used in a further LDA to optimise the separation between the treatment groups.

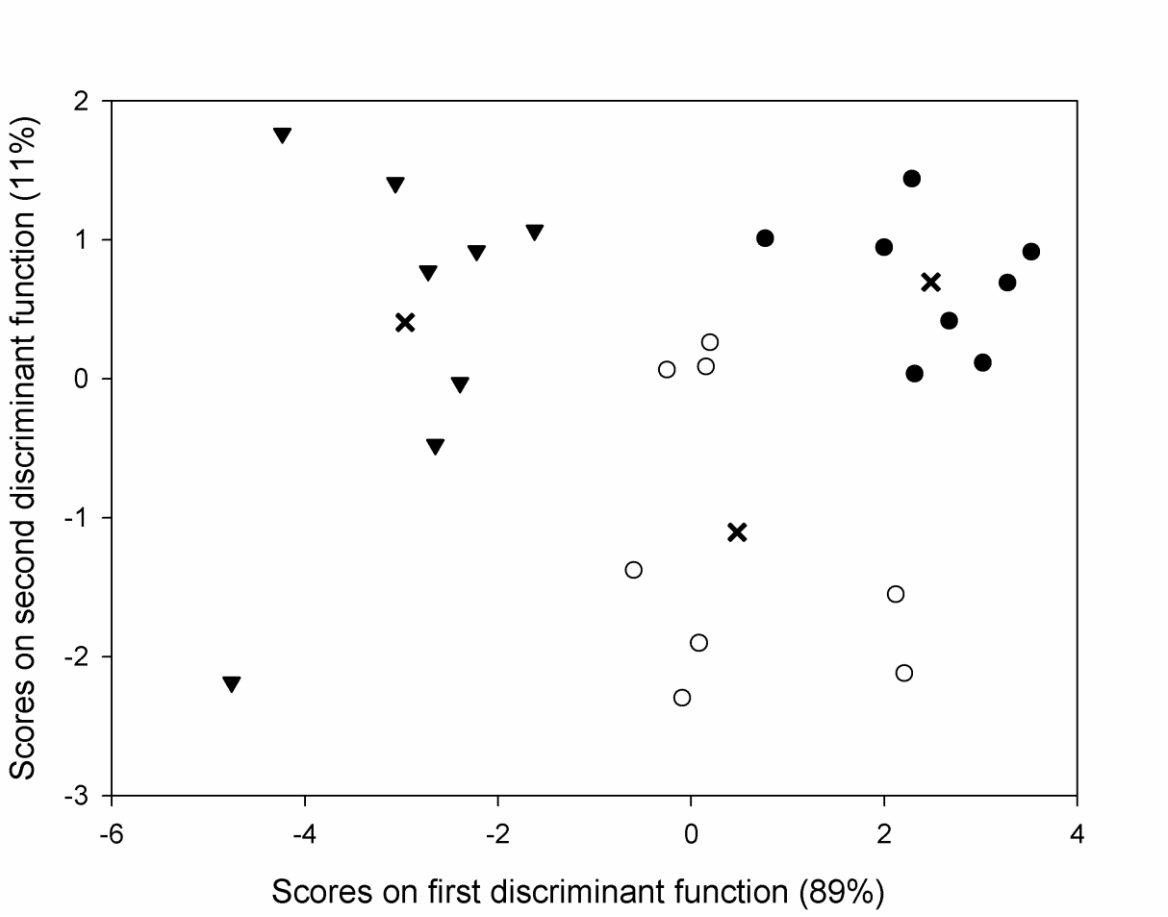


Figure 3.26. Plot of the scores on the two discriminant functions obtained from a linear discrimination analysis of the first five principal components (67% of the total variation) of the standardised spectral data from the lindane experiment. (closed circles - control; open circles - low dose of lindane; closed inverted triangles - high dose of lindane). The positions of the treatment means are indicated by crosses.

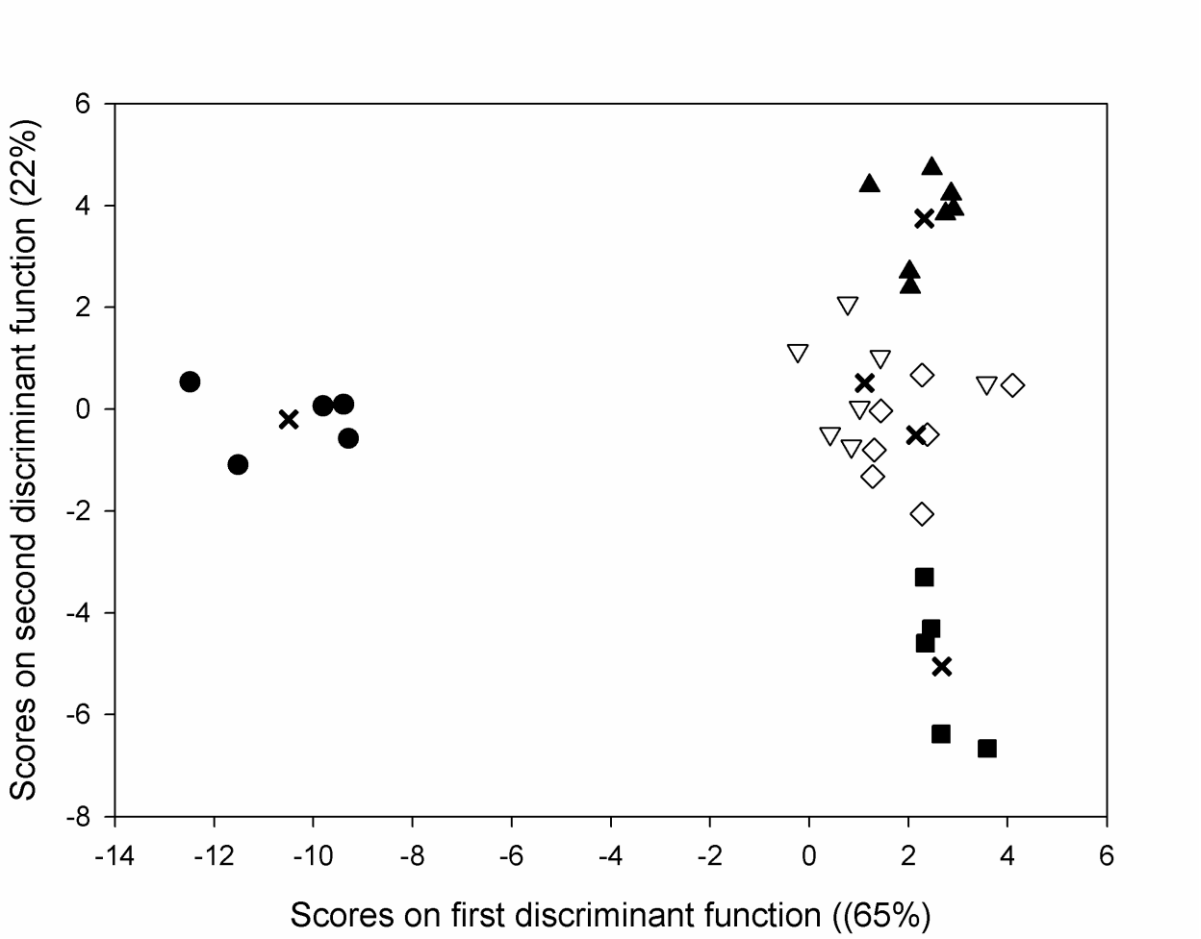


Figure 3.27. Plot of the scores on the first two discriminant functions obtained from a linear discrimination analysis of the first six principal components (93% of the total variation) of the standardised spectral data from the atrazine experiment (closed circles - hypoxia; open inverted triangles - control; closed squares - starvation; open diamonds - low dose atrazine; closed triangles - high dose atrazine). The crosses indicate the treatment means.

3.6. Conclusions

In the current study a clear separation between all of the treatment groups and controls was achieved for both toxicants where 100 % of group treatments were classified correctly. Further statistical analysis showed the robustness of this technique where permutation testing resulted in the less than 33 % of the group treatments classified correctly when the ‘y’ matrix was

randomised (all below this success rate for atrazine and 68 % for lindane). The complex interactions between the factors investigated made it difficult to identify specific metabolic affects caused by the treatments, there is evidence that lindane causes a general depression of activity, whilst atrazine stimulates metabolic activity and this is consistent with published biochemical and physiological data.

One significant difference between this investigation and that of (El-Shenawy et al., 2006) was the recording of no significant increase in ammonia levels (< 0.1 mg/L throughout) that were observed after 28 days for both lindane (5 fold increase) and atrazine (24 fold increase). One theory for this increase not being observed during this study is that the mussels were housed in 2 litre crystallising dishes open to the air and volatilisation. Whereas El-Shenawy used smaller closed vessels leaving little room for circulation of excretion products that could them build up if production was increased over the 24 hour renewal period.

M. edulis is a model organism that has been widely used for biomonitoring and as an indicator of pollution. This work using model compounds and a simple extraction procedure that yielded clean spectra in which metabolites from a range of classes could be identified indicates the potential utility of the metabolomic approach for assessing the potential impact of substances of emerging concern (e.g., pharmaceuticals) where mode of action in invertebrates is not known or uncertain.

Chapter 4

An environmental ^{1}H NMR metabolomic study of the toxicity of three insecticides, fenitrothion, methiocarb and permethrin, to *Chironomus riparius*

4.1. Introduction

The use of pesticides for crop protection and animal health applications (e.g. sheep dip) provides opportunities for some of the applied material to enter the aquatic environment through routes such as spray drift (Maltby and Hills, 2008), run-off events and leaching (Kreuger *et al*., 1999). These contaminants (especially hydrophobic chemicals) can become associated with aquatic sediments and persist within this environment (Leppanen and Kukkonen, 2006). These pose a threat to the health of non-target organisms found within both surface water and sediment. It is therefore important to monitor the effects of these contaminants on such organisms within both environmental compartments. The distribution between solid material (suspended solid and sediment) depends to a large extent on the polarity of the compound. This affects the availability to the organism. The route of entry to the organism is also affected by the polarity. Hydrophilic chemicals being retained in solution in the environmental medium, but biological membranes and epithelial layers have relatively low permeability to these compounds. The main routes of entry available to these substances are the respiratory surface, and the gut wall. More non-polar materials are present at lower concentrations in the water phase because of increased binding to solids, and lower water solubility. However, biological systems are permeable to these compounds, and partitioning favours accumulation in the lipids of organisms. Material adsorbed to particulate matter can also enter the metabolic pool of filter feeders and sediment feeders by ingestion (De Lange *et al*., 2005). It is because of these factors that sediment-water toxicity tests are carried out. One organism used

in such assays is the sediment dwelling larva of the non-biting midge *Chironomus riparius*. This species is a useful assay species because it is an aquatic insect that is widely distributed in the environment and is found in polluted areas. It is exposed to both the water column through ventilatory action, and the sediment in which it burrows, and hence it makes an ideal candidate for such toxicity tests (OECD, 2004a).

This study was to investigate the toxic actions of three insecticides (fenitrothion, methiocarb and permethrin) on the metabolism of the chironomid larva *Chironomus riparius*. Many toxicity assays are short term (acute) and use a range of endpoints including mortality, and some sub-lethal endpoints (e.g. changes in motility or feeding activity) (Callaghan et al., 2001, De Lange et al., 2005, Hirthe et al., 2001, Ibrahim et al., 1998). In the current work a short term exposure (24 h) was used to compare the effects of the toxicants added to the overlaying water using standard methods (OECD, 2004b).

4.1.1. Biology of Chironomus riparius

The family of the chironomidae are that of true flies (order Diptera) and are widely distributed and frequently the most abundant freshwater insects. Larval chironomids can survive at extremely low levels of dissolved oxygen and in some cases in these types of areas may be the only insects found in the benthic sediments. The adults are familiar as swarming insects around bodies of standing waters, while the larvae are known as ‘blood worms’ due to there vivid red colouration. The larval stages themselves are highly homogeneous

between species and high magnification is needed with a lot of expertise the separate the species by larval morphology alone. With only the pupal cast (exuviae) having consistent identification at lower magnifications. Although the species has been found in every conceivable freshwater environment, they are found predominantly in both lotic (running waters) and lentic habitats (standing waters) (Armitage et al., 1995).

4.1.1.1. Morphology; egg, instars, pupa & imago

Once mating has occurred within the swarming adults the females will lay their egg ropes attached to some substrate just under the waters surface. The egg ropes themselves in this species are arranged in a helical ribbon like fashion. The eggs themselves are contained within a protective gelatinous matrix with over 600 individual eggs per rope (fig. 4.1). Generally each adult will lay two egg ropes, with the second usually poorly formed and containing less eggs.

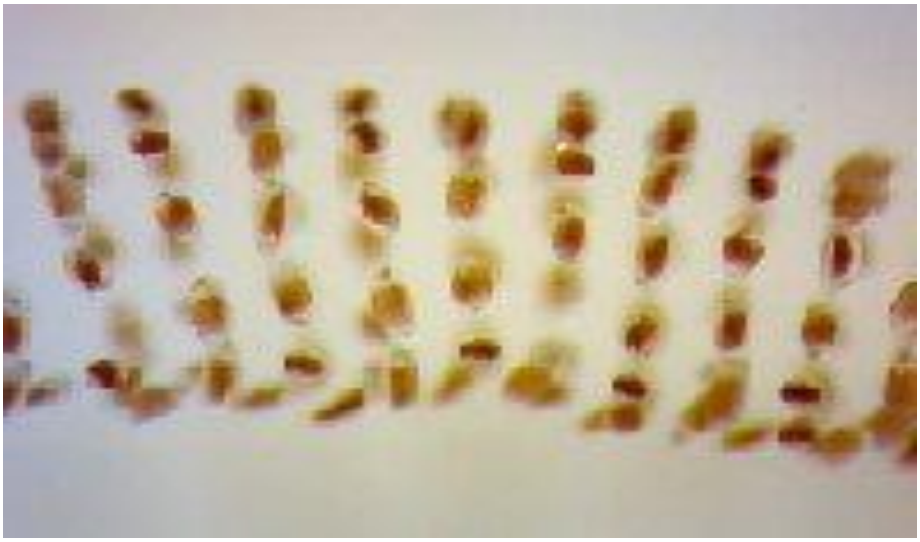


Figure 4.1. Chironomus riparius helical egg rope (50 x magnification)

Under ideal conditions the eggs will hatch after only a few days and the first instar (fig. 4.2) larvae migrate to the sediment surface. Once the sediment surface has been reached the larvae moult to the more recognisable second instar (fig. 4.3) having a well developed non-retractile head capsule with mandibles. The head capsule is sclerotized and of a fixed size compared to the rest of the body and splits during moulting, therefore this is used as a diagnostic marker for instar stage. The larval body contains three thoracic segments, followed by nine narrower abdominal segments. There are four larval stages with late fourth instar larvae recognisable by swollen thoracic segments and intense 'blood red' colouration (Cranston, 1997).

Figure 4.2. First larval instar of *Chironhomus riparius* (100 x magnification)

Figure 4.3. Second, third and fourth larval instars and pupal stage (20 x magnification)

The larvae oxygenate their blood directly from the water column across their cuticle, and therefore do not have specific breathing appendages. Whilst in their burrows the larvae pulsate their bodies to draw water through the tube tunnel, replenishing the boundary layer surrounding their bodies with fresh oxygenated water. The blood red colouration is formed from the high levels of haemoglobin that has a high affinity for oxygen. These are unlike those of vertebrates such as ourselves that has a low affinity for oxygen in a high oxygen tension environment. This allows the population to survive in extremely low level oxygen environments.

The short-lived pupal stage (usually less the 24 hours in this species) involves major changes in morphology during metamorphosis to the adult. The pupae live in the tubular burrows produced by the fourth instar until ready to hatch. At this point all adult features are visible within and the pupa ‘swims’ its way to the surface using full body jerking movements. The adults then emerge from the pupal skin or exuviae and these are readily used for species identification (see section 4.2.2.1.).

The adults are recognisable and are similar in appearance to mosquito’s, with a single pair of wings, six long legs and kidney shaped dichoptic eyes. The most distinguishing feature is that of the antennae that are sexually dimorphic, with the males displaying prominent plumose antennae.

4.1.1.2. Feeding behaviour

The species are deposit feeders, feeding on material deposited on the submerged sediments. They feed by extending their head and anterior body parts outside of the tube whilst the posterior segments maintain contact with the inner surface of the tube. Therefore foraging areas are restricted to the region surrounding the tube. The tube-building itself is initiated by the first or second instar larvae. The larvae will use whatever substrate is available to them and stick particulates together by applying salivary secretions. The larvae will then crawl through the construct to form a tunnel. Under conditions of stress the organisms will remain inside their tubular burrows until favourable conditions return or starvation forces them to emerge (Berg, 1997). The adult chironomids are non-biting midges (do not feed on blood), and the mandibles are termed reduced as the adults are short-lived (>7 days) feeding on nectar if feeding occurs at all (Cranston, 1997).

4.1.1. Ecotoxicology of fenitrothion, methiocarb and permethrin

The insecticides in this study represent those currently widely used in the control of pest species (Ibrahim *et al*., 1998). The organophosphate (OP) insecticide fenitrothion inhibits cholinesterases and carboxylesterases by binding with the active site of the enzymes (Damasio *et al*., 2007). The carbamate, methiocarb is also an acetylcholine esterase inhibitor. Both compounds produce a build up of acetylcholine in the synapse, and this disrupts normal nervous system function (Habig and DiGiulio, 1991).

Permethrin is a synthetic pyrethroid and acts as an axonal poison acting on cation channels, in particular the sodium channel, and causes changes in the permeability of nerve membranes to cations. This leads to the production of repetitive discharges. The increased frequency of action potentials that is also seen in the post synaptic fibres in carbamate and organophosphate poisoned nervous systems leads to a loss of control of motor functions. All three of the insecticides produce similar whole body effects of hyperactivity, tremors, paralysis and death (Hassall, 1990).

Due to this similarity of symptoms of toxicity classical toxicological assays would only give limited information on secondary effects of the primary lesions. Enzyme biomarker studies of the OP insecticide pirimiphos-methyl found that for 48 and 96 h exposures (10 ng g^{-1}) acetylcholine esterase activity was significantly reduced in exposed larvae. This led to adverse developmental and reproductive effects (Crane *et al*., 2002). A study of honey bees exposed separately to cypermethrin and fenitrothion showed that both induced a decrease in the activity of ATPase and acetylcholinesterase, and related this activity to general metabolic perturbations, though exact effects were not measured (Bendahou *et al*., 1999).

Other studies have shown that exposure of 4^{th} instar *Chironomus riparius* larvae to three insecticides fenitrothion, cabaryl and carbofuran produced a significant reduction in the activity of acetylcholine esterase. Analysis of amino acids in extracts of treated larvae showed a difference between the secondary effects of the OP and carbamates. Alanine was accumulated in the larvae

treated with fenitrothion relative to the animals treated with methiocarb (Forcella *et al*., 2007). This study shows the advantage of investigating the metabolite content of these organisms, although this group had to select the metabolites to investigate. The advantage of the metabolomic approach is that the whole extractable metabolic pool is measured (Viant et al., 2003).

The three pesticides are all found in freshwater environments above the standard regulatory level of 0.1 $\mu g\ L^{-1}$. An extensive study of the Humber river system found levels of permethrin at 0.3 $\mu g\ L^{-1}$, and for fenitrothion at 0.2 $\mu g\ L^{-1}$ in surface waters of the Calder system during 1995-96, July-May. Sediment levels were also recorded and found to be much higher for permethrin at over 10 000 $\mu g\ Kg^{-1}$ while fenitrothion levels were below 100 $\mu g\ Kg^{-1}$ during the same test period (House et al., 1997). The carbamate methiocarb was discovered as high as 5.4 $\mu g\ L^{-1}$ in ground water of the Yagin Valley, Mexico (García de Llasera and Bernal-González, 2001). As these levels of the pesticides are detected in the aquatic environment it shows the importance of understanding the effects these sublethal levels have on the population ecology of our water systems.

Therefore the utilisation of a ^{1}H NMR metabolomic analysis to analyse the metabolites in larvae exposed to sub-lethal concentrations of the three insecticides (fenitrothion, 1 $\mu g\ L^{-1}$, methiocarb, 1 $\mu g\ L^{-1}$ and permethrin, 0.1 $\mu g\ L^{-1}$) was investigated. Perturbations of levels of endogenous metabolites relative to controls and to each other were measured. This approach may identify potential biomarkers of toxicity and show a separation between the

secondary effects of the three test compounds. Previous metabolomic studies have been used successfully to identify biomarkers of exposure of terrestrial species such as the earthworm to toxicants. Using this approach (Gibb et al., 1997b) identified free histidine as a novel biomarker of the exposure of earthworms to copper. It was suggested that the histidine may be acting as an energetically 'low cost' substrate for metal ion detoxification.

(Bundy et al., 2001) studied the metabolic effects of exposure of earthworms to a model compound 3-fluoro-4-nitrophenol. They used one- and two-dimensional ^{1}H NMR spectroscopy to analyse small metabolites in coelomic fluid. The data were analysed using multivariate methods that allowed the characterisation of the metabolite profiles in treated and control animals, and the identification of changes associated with the toxic insult. Concentrations of acetate and malonate were decreased in treated animals whilst concentrations of succinate were increased relative to the controls. These workers postulated that these changes indicated that this toxicant caused an increase in the energy flow into the anaerobic pathway by interference with carbohydrate metabolism.

This current study has used metabolomic methods to evaluate the effects of sub-lethal concentrations of three test compounds (fenitrothion, methiocarb, and permethrin). Appropriate no observable lethality levels (NOLL) were determined using classical toxicological assay techniques, and these sub lethal concentrations were used in the subsequent metabolomic experiments. Larvae were exposed to these insecticides in the water column over a short

(24 h) period. The impacts of these low exposure levels on the biochemical pathways were then investigated to identify possible biomarkers of toxicity. The aim was to see if it was possible to distinguish between the secondary effects of the three insecticides on metabolism.

4.1.2. Aims

- To investigate the potential of metabolomic methods for identifying the impact of individual toxicants in the aquatic organism *Chironomus riparius* by three model toxicants, fenitrothion, methiocarb and permethrin.
- To identify the species of the model organism using pupae exuviae identifiers and optimise the extraction of the small molecular weight metabolites from the tissue matrix of *Chironomus riparius* with minimal interference from proteins and lipids.
- To utilise one and two dimensional NMR spectroscopy coupled with metabolite addition experiments to identify the major peaks within the metabolic profile of the test organism and quantify the metabolic changes observed.
- Utilise robust and validated multivariate statistical methods to identify the patterns of metabolic change and the associated metabolites.

4.2. Materials and methods

4.2.1. Chemicals and reagents

The insecticides, fenitrothion, methiocarb, permethrin (pure analytical standards), and amino acids were purchased from Sigma Aldrich (Poole, UK). All solvents (chromatographic grade), salts (Analar grade), and acids (Analar or better grade) were obtained from Fisher Scientific UK Ltd. (Loughborough, UK). Deuterium oxide [99.6% (v/v)] was purchased from Goss Scientific Instruments Ltd (Nantwich, UK). Stock solutions (1 mg L^{-1} each) of the three insecticides were prepared in acetone. All of the water used in the laboratory was de-chlorinated tap water. In order to remove chlorine the water was left in a 20 L glass tank with a small activated carbon filter unit (Hydor pico pump) and left under UV light for 24 h prior to use. The water was oxygenated to greater than 8 mg L^{-1} (90% saturation)] using a Maxima™ pump (Hagan Ltd, UK) and an air stone. Water quality (pH, ammonia, and nitrate) was checked using Dry-Tab® tablet reagents (Aquarium Pharmaceuticals, Chalfont, Pennsylvania, USA) dissolved in a 10 mL sample of the tank water. The chironomids were supplied by AstraZeneca (Environmental Laboratory, Brixham, UK) in the form of egg ropes from a culture of a known, single species (*Chironomus riparius)* originally supplied by Dr David Pascoe, (School of Biosciences, Cardiff University, UK) and the eggs used to set up a culture. The diet of the chironomid larvae was Tetra Min® fish food obtained from Maidenhead Aquatics (Havant, UK), and that of the adult stage was a sucrose solution (0.01 g mL^{-1}).

4.2.2. Culture of *Chironomus riparius*

It is important for toxicity testing that the chironomids used have a known history, are laboratory bred, and healthy (section 2.10)(OECD, 2004a). The culture was maintained in a custom built Perspex box (80 cm high, 80 cm wide, 40 cm deep) with a wire mesh (60 x 20 cm) slot front and back that allowed airflow whilst retaining the adult flies. The larval cultures were maintained under semi-static conditions (water replaced weekly or more frequently if water quality deteriorated) at 20 ± 2 ºC in a temperature controlled room, under a cool white fluorescent light with a photoperiod of 16/8 h (light/dark). At least three separate stock cultures, staggered in age, were maintained at any one time to ensure that adults were always present in the culture tank.

The larval cultures were maintained in crystallising dishes (2 L, Fisher Scientific UK Ltd) containing approximately 1 cm depth of acid washed, low iron laboratory sand (40-100 mesh, Fisher Scientific UK Ltd) as a substrate. The overlying water (1500 mL) was dechlorinated tap water maintained at 20±2 ºC, pH of between 7-9 and a conductivity of approximately 555 µS cm^{-1}. Dissolved oxygen was maintained above 75 % of the air saturation value by bubbling air through narrow bore glass pipettes. This arrangement minimised disturbance of the sediment surface. The water hardness, pH and ammonia concentration were monitored regularly using tablet reagents in 10 mL of

culture water. The ammonia concentration was kept below 0.4 mg L^{-1}. If water quality was found to have deteriorated, then the overlying water was replaced.

The cultures were fed every 3 days with finely ground fish food (Tetra Min®). The amount of food varied with stage of development and feeding rate of the larvae: approximately 400 mg of food was the largest amount given, and this was reduced for the smaller 1st and 2nd instar larvae that require less food than later stages of development. When the larvae start to pupate feeding rates fall. Where excess food remained on the substrate surface the amount of food provided was reduced to avoid bacterial growth, water discolouration, and an increase in the ammonia concentration in the water, since these can lead to larval mortality. Waste accumulated on the surface of the substrate was removed by gentle stirring to lift it into the water column that was then immediately siphoned off from approximately 0.5 cm above the sediment surface. Fresh medium was then added carefully to avoid disturbance of the sand.

Once the adults had emerged the discarded pupal cuticles were removed from the culture using a wide bore pipette. Adults were fed on the sucrose solution soaked into a cotton wool pad in order to maximise their life span that is typically 4-5 days (Armitage et al., 1995) and to increase their potential for mating. A minimum height of 60 cm was maintained above the water in the culture dishes in order to provide sufficient space for the adults to swarm during aerial mating. Egg ropes that were deposited in the culture dishes were removed using a wide bore pipette and then each was retained separately in a

well of a plastic culture tray with approximately 5 mL of water. The trays were covered with a lid, labelled with date of collection and kept under the same environmental conditions as the larval cultures for 24 h. Only healthy larvae emerging within 24 h of egg laying were used in this study. Hatched larvae from each egg rope, up to 600 individuals, were used to maintain the cultures or to provide samples for toxicity testing. The latter were maintained under the standard culture conditions until they reached the 4th instar, when they were transferred (using a wide bore pipette) to separate dishes for the toxicity assays.

*4.2.2.*1. Chironomid species identification

The larva of *Chironomus riparius* (Diptera, Chironomidae), a non-biting midge, was used as a model freshwater organism. These larvae, known as 'blood worms', have few obvious external distinguishing features, and there are many hundreds of species making them difficult to identify. For this particular study the organisms were supplied by Astra Zeneca (Berkshire, UK) as egg ropes from an already identified population. However, for the purpose of this study this identification was confirmed using microscopal examination and a key (Wilson and Ruse, 2005) that uses identifying features from the morphology of the pupal exuviae that are discarded as the adults emerge from the water. The exuviae have several morphological features that can be used to identify the various species. Thoracic horns are organs used for respiration by the pupae and are found each side of the thorax (Fig. 4.1). There are nine abdominal segments (numbered from the thorax down (I-IX)), and each of the

abdominal dorsal cuticular plates (the tergites) has distinguishing features that can be used for purposes of identification. Two important features are found on tergite VIII and IX. Tergite VIII carries a spur (Fig. 4.2) that can exhibit many different spinal forms, and tergite IX bears the anal lobes that carry hair fringes and other distinguishing features (Fig. 4.3 and 4.4).

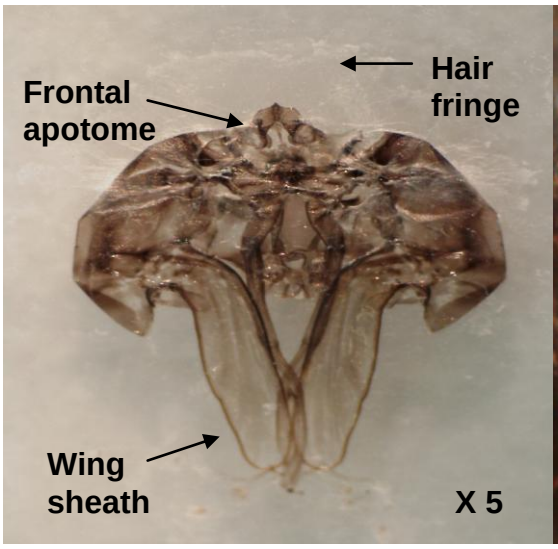


Figure 4.1. Thorax segment of pupal exuviae with the wing sheath clearly visible and surrounded with a hair fringe, horns not visible.

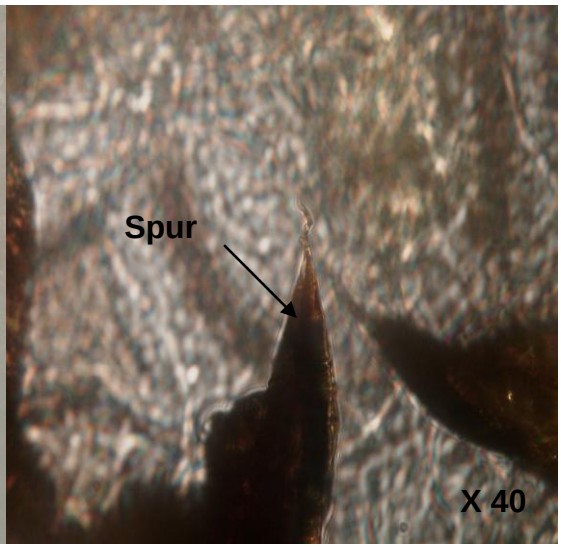


Figure 4.2. Segment VIII spur with group of two spines fused at the base.

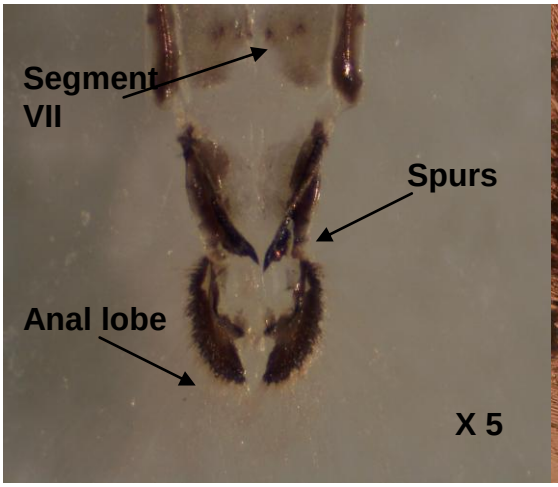


Figure 4.3. Anal lobe and segments VII and VIII carrying the spurs.

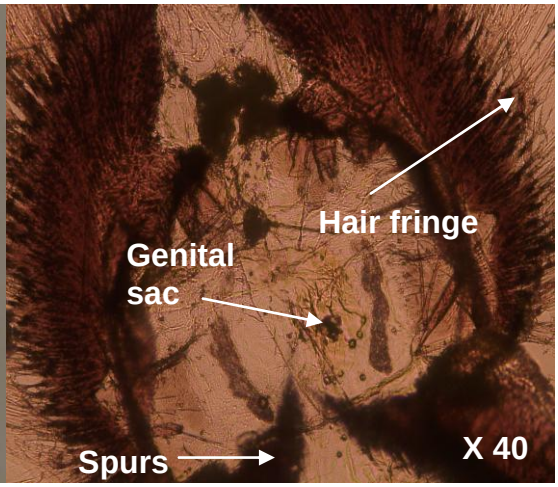


Figure 4.4. Anal lobe with genital sac and fringe hair features

4.2.3. Assays to assess toxicity of insecticides

Concentrations of insecticides in environmental waters tend to be low and it was therefore necessary to identify suitable sub-lethal concentrations for use in this metabolomic study. Bioassays were conducted, and on the basis of previous short term (24 or 48 h) exposure studies for permethrin (Stephenson, 1982, , Ibrahim et al., 1998, Conrad et al., 1999), fenitrothion (Choi *et al*., 2001, Choi *et al.*, 2002, Choi and Roche, 2004, Forcella *et al*., 2004) and methiocarb (Karnak and Collins, 1974, Pery *et al*., 2003b). The following concentration ranges of the insecticides were selected: 0, 1.0, 2.0, 4.0, 8.0 μg L^{-1} for fenitrothion and methiocarb, and 0, 0.1, 0.2, 0.5, 1.0 μg L^{-1} for permethrin. The assays (24 h exposure) (OECD, 2004a) were carried out under static conditions in 150 mL crystallising dishes (Fisher Scientific UK Ltd) with each dish containing 75 g of low-iron sand and 125 mL of dechlorinated water. During the bioassays animals were maintained under the same conditions as described above (Section, 4.2.2) for the culture of these animals, but were not fed for 24 h prior to the assays and during the exposure in order to encourage the exhibition of foraging behaviour. Three replicates, each with 10 larvae, were used for each concentration. The behaviour of the larvae was noted at the end of the exposure period.

4.2.4. Insecticide exposures for metabolomic study

The 24 h exposures to the insecticides were carried out under the same conditions as those used for the culture of the larvae (Section 4.2.2) and for the toxicity assays (Section 4.2.3). Ten 4^{th} instar larvae were used for each replicate exposure for each insecticide, and were not fed during the exposure period to minimise the gut contents since it is not possible to remove these prior to homogenisation for metabolomic studies. Six replicate sets of ten larvae were used for each insecticide, five to be used for tissue extraction and a spare set in case any of the individuals died, became immobile or went into pupation during the exposure. Any unusable individuals were replaced with a healthy larva from the reserve test vessel so as to ensure 10 specimens were available for metabolite extraction. The three insecticide test solutions (fenitrothion and methiocarb 1.0 $\mu g\ L^{-1}$, and permethrin 0.1 $\mu g\ L^{-1}$) were prepared from stock solutions (1 mg L^{-1} in acetone). Fresh test solutions were prepared from the stock solutions 12 h before use in the dishes to allow them to equilibrate with the experimental temperature (20 °C). The acetone was used to aid solvation and ensure uniform distribution of the test chemicals in the test vessels, but the volume was minimised to avoid any toxic effects. Controls were exposed to the highest concentration of acetone as used in the insecticide exposures (10^{-4} % v/v). The spent water was filtered through activated charcoal before disposal.

4.2.5. Tissue extraction

After the 24 h exposure, the larvae were removed from the test vessel using a wide bore pipette, washed in distilled water, blotted dry on paper tissue, wrapped in aluminium foil and immediately plunged into liquid nitrogen to stop metabolism. The sets of ten larvae were then homogenised and extracted using the method described in section 2.3. With the exception that 10 mg (instead 100 mg in mussels) of dry weight material was used for each sample.

4.2.6. One-dimensional ^{1}H NMR spectroscopy

The same method was used as mentioned in section 2.6 for the proton spectral analysis. This and all subsequent two-dimensional spectroscopy were performed on a Unity Inova 600 NMR spectrometer (Varian Ltd, Oxford, UK) equipped with a cryoprobe held at 25 K. The spectrometer was operating at a spectral frequency of 599.80 MHz at 298 K throughout.

4.2.6.1. Two-dimensional ^{1}H-^{1}H NMR total correlation spectroscopy (TOCSY)

Two-dimensional (2-D) ^{1}H-^{1}H NMR spectra were obtained using a tnTOCSY pulse sequence (Fig. 4.5) incorporating a pre-solvent saturation pulse with a 1.5 s saturation delay and an acquisition time of 0.300 s. Spectra were recorded with a spectral width of 13.34 ppm in direct and indirect dimensions.

128 transients were collected over a time period of ~13 h into 16,824 data points in the F1 dimension and 4096 data points in the F2 dimension. Fourier transformation was applied with an exponential line broadening function of 5.00 Hz in the F1 dimension and 11.00 Hz in the F2 dimension and further sine-bell functions to both dimensions. All spectra were phase and baseline corrected in VNMR software and calibrated to the internal standard TMSP at 0.00 ppm.

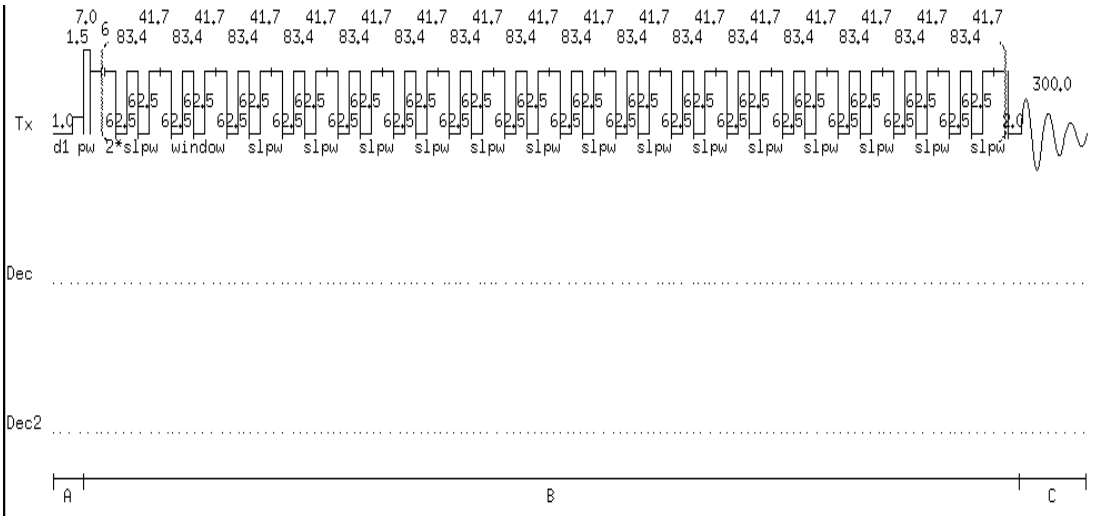


Figure 4.5. Standard tnTOCSY pulse sequence.

4.2.6.2. Two-dimensional homonuclear ^{1}H decoupling NMR spectroscopy

A two-dimensional homonuclear ^{1}H decoupling pulse sequence was used (Fig. 4.6) with an acquisition time of 7.5 s, sweep width of 13.34 ppm and a pulse width of 6.0 s proceeded by a 1.0 s relaxation delay. A saturation delay of 1.5 s was set at a power of 6.00 dB at the saturation frequency of water (–218.6 Hz, this is the frequency offset corresponding to the water frequency at 4.774 ppm) to reduce the residual water peak. An exponential line

broadening function of 0.25 Hz was applied with a minimum of 4 and maximum of 256 transients collected into 524,288 data points.

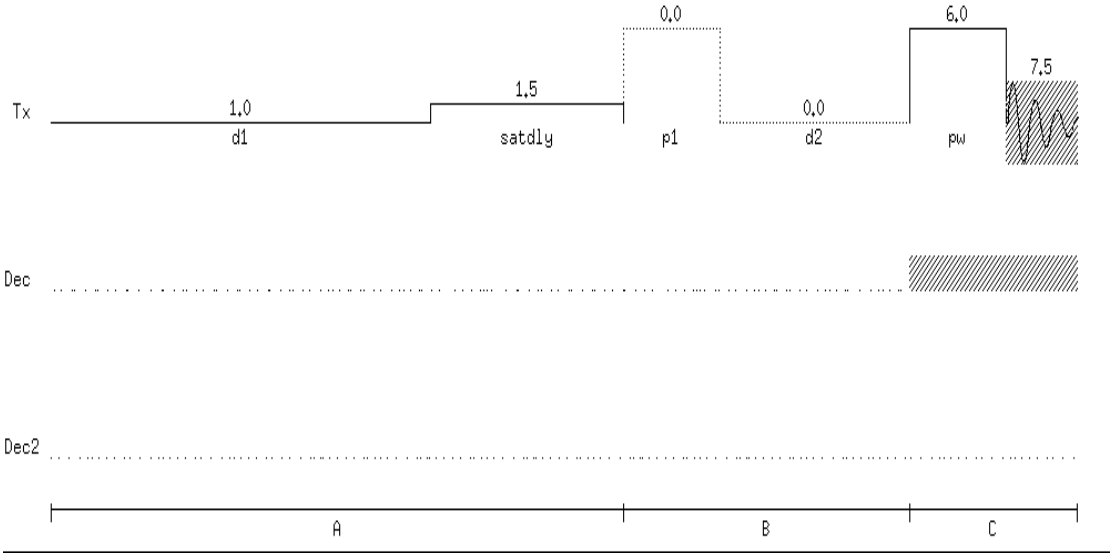


Figure 4.6. Homo-decoupling pulse sequence, shaded area indicated decoupling.

4.2.6.3. Two-dimensional heteronuclear ^{1}H-^{13}C NMR spectroscopy (cHSQC)

A two-dimensional heteronuclear ^{1}H-^{13}C pulse sequence was applied (Fig. 4.7) with an acquisition time of 0.073 s, sweep width of 10.82 ppm and pulse width of 7.0 s. The frequency for carbon was 150.84540 MHz sweep width of 140 ppm, pulse width of 14.40 µs, and 128 transients were collected over a time period of ~14 h.

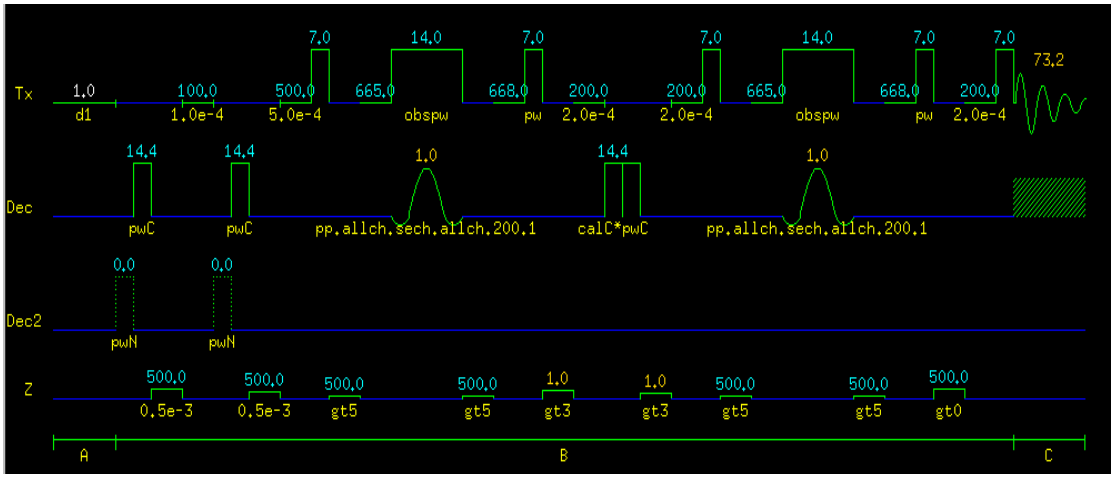


Figure 4.7. Standard carbon HSQC pulse sequence, with carbon decoupling (Dec), z indicates the set of gradients used.

4.2.7. Spectral pre-processing and statistical analysis

The processing and analysis used are in section 2.6, with a matrix size of 20 samples by 823 bins, this single set comprised of all the chemical stress experiments. The samples by bins matrix was transposed to give the samples in rows and variables in columns.

4.3. Results and discussion

The metabolic profile of the test species identified as *Chironomus riparius* (see section 4.2.2.1.) was investigated by use of one- and two-dimensional NMR techniques mentioned above. In this study it was shown that the insecticides, fenitrothion, methiocarb and permethrin at no observed lethality levels (NOLL) concentrations produced changes in the metabolic profiles of the treated larvae relative to the control animals, and relative to each other.

4.3.1. Toxicity assays of insecticides

Counts were made of organisms exhibiting symptoms of stress (burrowing into the sediment), and those exhibiting no stress response (remaining on the surface in search of food). Any dead or immobile individuals were counted (Table 4.1). The LC_{50} was estimated for each compound and the values (with 95% fiducial confidence intervals) were 3.0 (2.5–3.6) $\mu g\ L^{-1}$ for fenitrothion, 7.6 (6.0-11.4) $\mu g\ L^{-1}$ for methiocarb, and 0.27 (0.22-0.32) $\mu g\ L^{-1}$ for permethrin. The NOLL for the three compounds were 1.0 $\mu g\ L^{-1}$ for fenitrothion and methiocarb, and 0.1 $\mu g\ L^{-1}$ for permethrin, and proportions of larvae exhibiting the stress response were 83% in fenitrothion and methiocarb, and 97% in permethrin in these concentrations. The NOLL were used in subsequent metabolomic experiments.

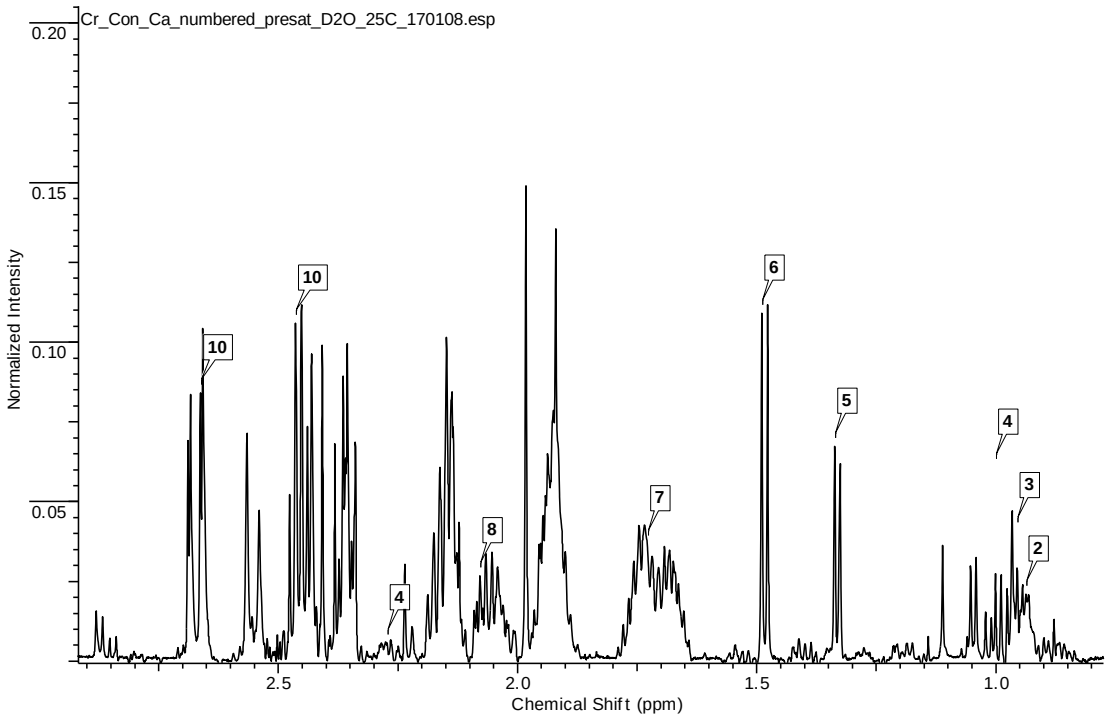
Table 4.1. Counts of chironomid larvae displaying avoidance behaviour (avoid) and the number of dead organisms used to identify a NOLL for further metabolomic studies.

Pesticide	Concentration $\mu g\ L^{-1}$	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
		Avoid	Avoid	Avoid	Dead	Dead	Dead
fenitrothion	0	0	1	0	0	0	0
	1	8	8	9	0	0	0
	2	7	8	7	3	1	3
	4	1	4	2	9	6	8
	8	1	0	1	9	10	9
methiocarb	0	0	0	0	0	0	0
	1	8	8	9	0	0	0
	2	9	9	10	1	0	0
	4	8	7	8	2	1	2
	8	5	4	4	4	6	6
permethrin	0	0	0	1	0	0	0
	0.1	10	9	10	0	0	0
	0.2	6	7	7	3	2	4
	0.5	1	2	0	9	8	10
	1.0	0	0	0	10	10	10

4.3.2. Metabolic profiles of healthy 4th instar larvae

Significant peak overlap (especially between 4.5 and 3.0 ppm (Fig. 4.8 a-c) was observed in the one-dimensional spectrum of a set of ten healthy 4th instar larvae, pooled to give sufficient tissue for metabolite extraction. Assignments were confirmed by using two-dimensional NMR experiments that resolved the overlapping peaks, and others within the spectrum (sections 4.2.6.1/2/3). Using these methods the peaks of thirteen metabolites, twelve amino acids and lactate (Table 4.2) and a further 82 unassigned peaks (Table 4.3) were resolved (Figures 4.9 and 4.10).

a


Cr_Con_Ca_numbered_presat_D2O_25C_170108.esp
Normalized Intensity
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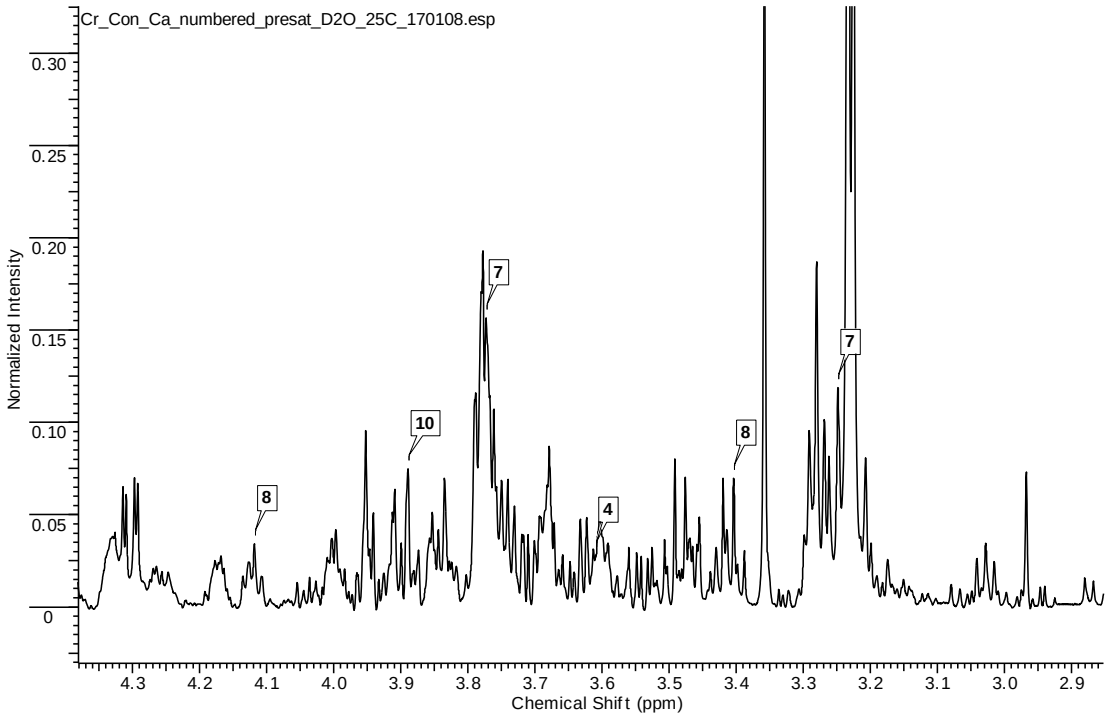
0.15
0.10
0.05
10
10
6
5
4
3
2
7
8
4
2.5
2.0
1.5
1.0
Chemical Shift (ppm)

b


Cr_Con_Ca_numbered_presat_D2O_25C_170108.esp
Normalized Intensity
0.30
0.25
0.20
0.15
0.10
0.05
0
7
7
10
8
8
4
4.3
4.2
4.1
4.0
3.9
3.8
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3.1
3.0
2.9
Chemical Shift (ppm)

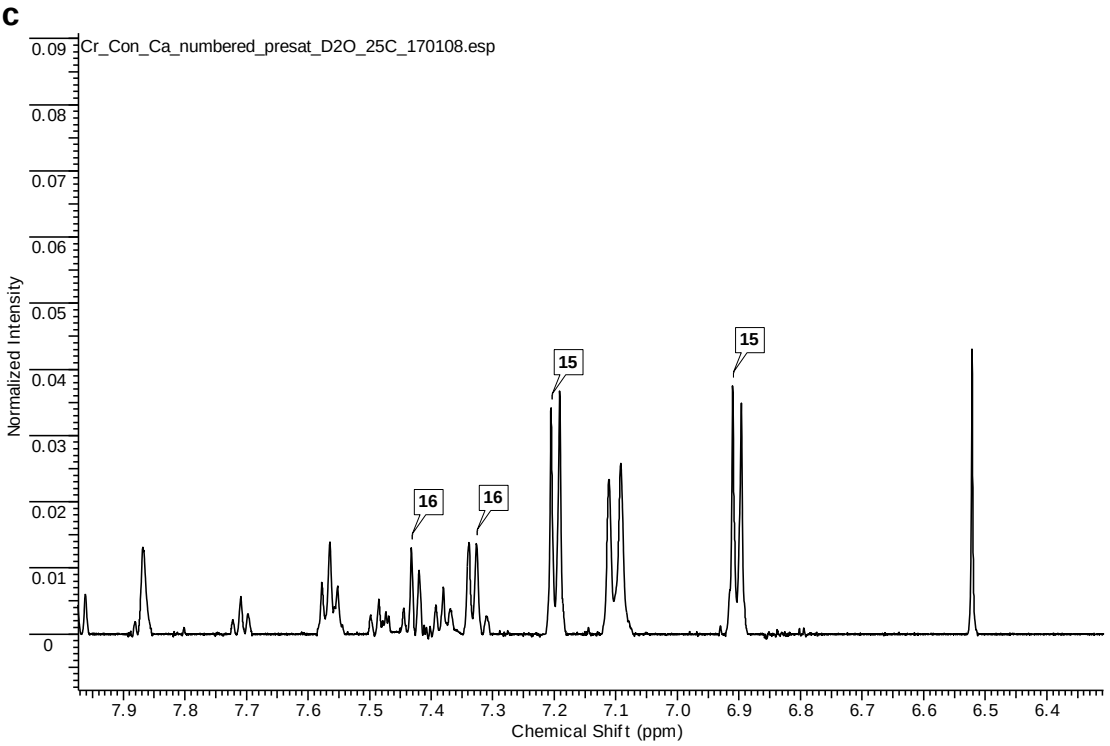

Figure 4.8. a-c. Representative one-dimensional ^{1}H NMR spectrum of whole body homogenates of *Chironomus riparius* larvae. The figure has been enlarged into three sections for better clarity with a. and b. representing the aliphatic region and c, the aromatic region with identified metabolite peaks numbered. Key to metabolites: 2. isoleucine, 3. leucine, 4. valine, 5. lactate, 6. alanine, 7. Arginine/phosphoarginine, 8. glutamate, 10. aspartate,15. tyrosine, 16. phenylalanine.

The ^{1}H NMR spectra displayed unique characteristics for this species when kept under ideal healthy conditions (section 4.2.2). A control group of spectra from a healthy population of these organisms was created so it was possible to detect any metabolic variations due to any physiological changes within the organisms resulting from exposure to toxicants (Bundy et al., 2001, Gibb et al., 1997b). As the organisms were pooled (ten organisms) to give a sufficient amount (10 mg dry weight) of tissue for the extraction, any metabolite variations between individuals would be significantly reduced.

Table 4.2. List of metabolites and their NMR assignments of both proton and carbon species identified in chironomid tissue.

Metabolites	Proton species, chemical shift (ppm) and peak multiplicity	Carbon species and chemical shift (ppm)	Couplings confirmed (method)
Amino acids			
Alanine	ha, 3.784 (q), hb, 1.483 (d)	ca, 51.776, cb, 17.408	ha-hb (tocsy)
Arginine/phosphoarginine	ha, 3.773 (t), hb, 1.920 (m), hg1, 1.660 (m), hg2, 1.731 (m), hd, 3.243 (t)	ca, 55.449, cb, 28.775, cg, 25.130, cd, 41.755	ha-hb, hg1-hd, hg2-hd (tocsy)
Asparagine	ha, 4.008 (dd), hb2, 2.958 (dd), hb1, 2.858 (dd)	ca, 52.487, cb, 35.775	ha-hb2, ha-hb1(hsqc)
Aspartate	ha, 3.903 (dd), hb2, 2.822 (dd), hb1, 2.676 (dd)	ca, 53.500, cb, 37.729	ha-hb22,hb21, ha-hb12,hb11 (tocsy)
Glutamate	ha, 3.761 (m), hb2, 2.129, hb1, 2.064, hq 2.354 (m)	ca, 55.772, cb, 28.275, cg, 34.767	ha-hb2, hb1, hg-hb2,hb1 (tocsy)
Glutamine	ha, 3.768 (t), hb, 2.136 (m), hq, 2.449 (m)	ca, 55.546, cb, 27.471, cg, 32.080	ha-hb, hb-hg (tocsy)
Isoleucine	ha, 3.675 (d), hb, 1.987 (m), hg11, 1.267 (m), hg12, 1.478 (m), hg2, 1.015 (d), hd, 0.944 (t)	ca, 60.720, cb, 37.162, cg1, 25.640, cg2, 15.893, cd1, 12.327	hb-ha, hb-hg2, hg11-hd1, hg12-hd1 (hsqc)
Leucine	ha, 3.737 (t), hb2, 1.736 (m), hb1, 1.689 (m), hg, 1.721 (m), hd2, 0.972 (d), hd1, 0.961 (t)	ca, 54.734, cb, 41.094, cg, 25.239, cd2, 23.244, cd1, 22.129	ha-hb1,hb2, hg-hd2,hd1 (tocsy)
Lysine	ha, 3.761 (t), hb, 1.908 (m), hg, 1.490 (m), hd, 1.733 (qn), he, 3.028 (t)	ca, 55.567, cb, 31.110, cg, 22.810, cd, 27.588, ce, 40.330	ha-hb, hg-he, hd-he (tocsy)
Phenylalanine	hd, 7.332 (d), he, 7.432 (t), hz, 7.379 (t), ha, 3.999 (dd), hb2, 3.290 (dd), hb1, 3.128 (dd)	cd, 128.494, ce, 130.400, cz, 130.069 ca, 55.567, cb, 37.560	hd-he, he-hd, hz-he, ha-hb22, hb21, ha-hb12,hb11 (tocsy)
Tyrosine	hd, 7.198 (d), he, 6.909 (d), ha, 3.944 (dd), hb2, 3.204 (dd), hb1, 3.128 (dd)	cd, 131.963, ce, 117.086, ca, 57.253, cb, 36.792	hd-he, he-hd, ha-hb22,hb21, ha-hb12,hb11 (tocsy)
Valine	ha, 3.6.14 (d), hb, 2.281 (m), hg2, 1.047 (d), hg1, 0.995 (d)	ca, 61.599, cb, 30.330, cg2, 19.169, cg1, 18.033	ha-hb (hsqc), hb-hg2, hb-hg1 (tocsy)
Organic acids			
Lactate	ha, 4.112 (q), hb, 1.330 (b)	ca, 69.742, cb, 21.286	ha-hb (tocsy)

Key: s, singlet, d, doublet, t, triplet, q, quartet, qn, quintet and dd, double doublet, h, proton, c, carbon.

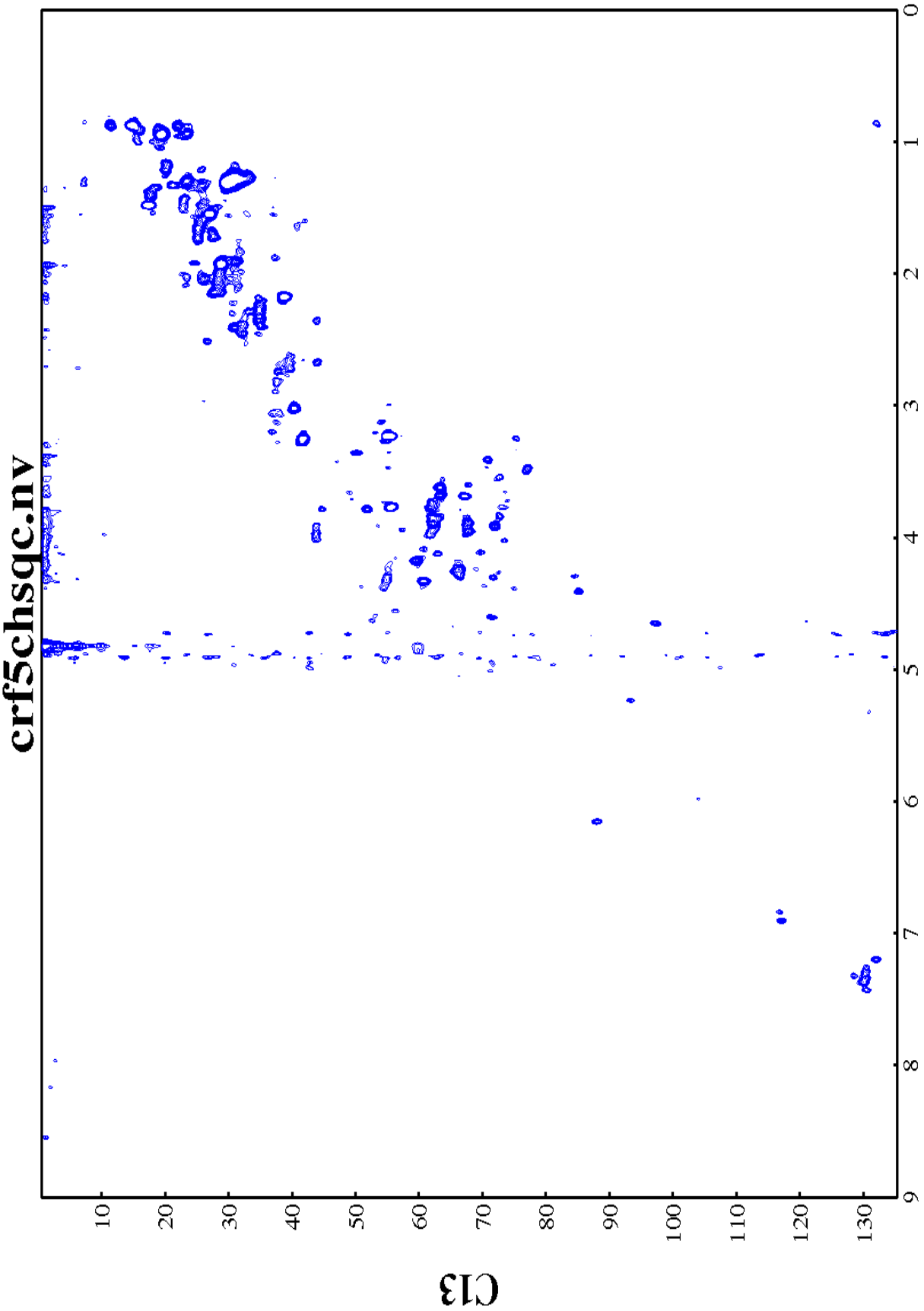

Figure 4.9. Representative two-dimensional cHSQC of whole body homogenate of *Chironomus riparius* larvae.

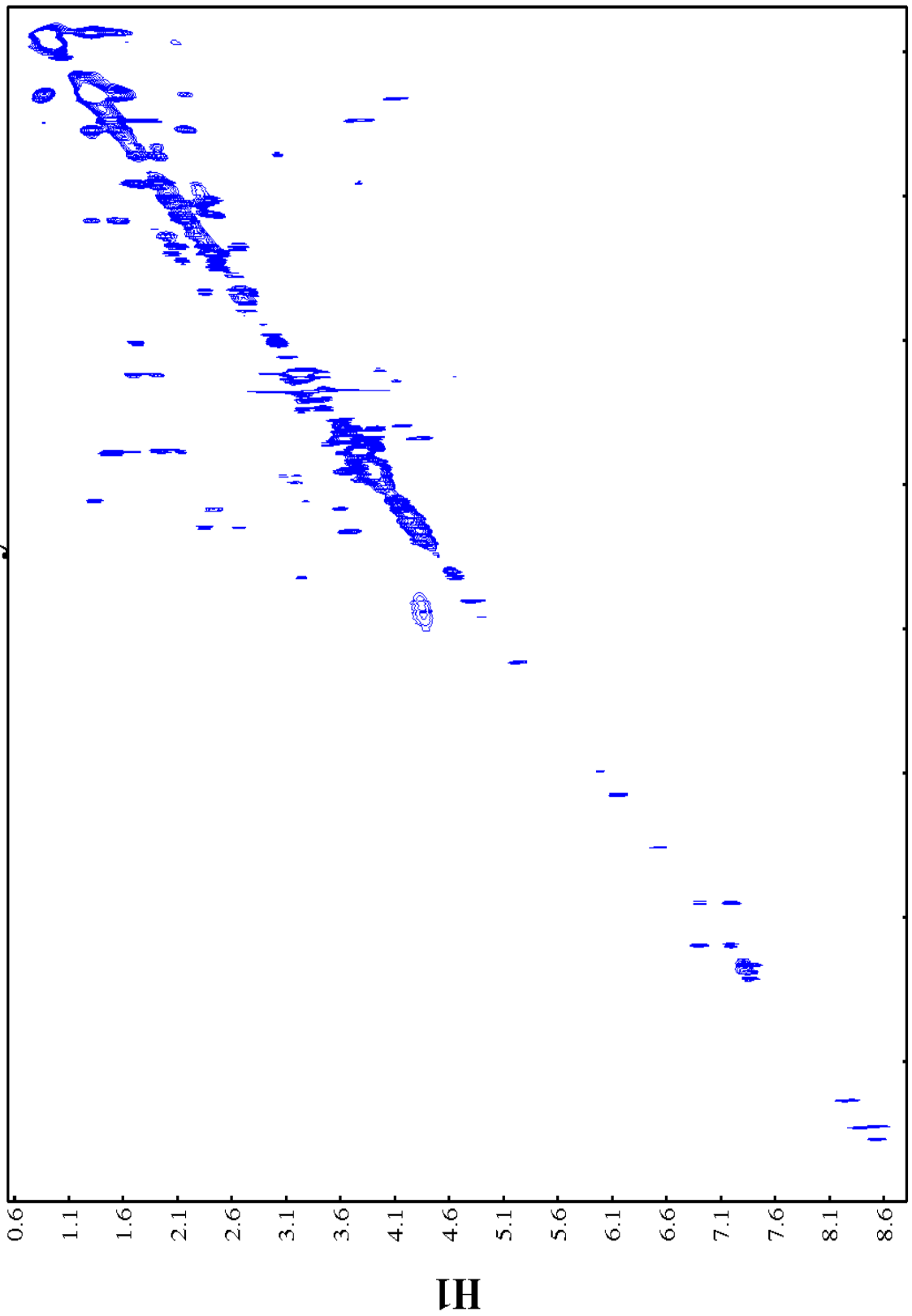

Figure 4.10. Representative two-dimensional TOCSY of whole body homogenate of *Chironomus riparius* larvae.

Table 4.3. Table of unidentified peaks in chronomid spectra with associated proton and carbon couplings.

Title	Proton ppm	Multiplet	HSQC carbon	TOCSY	HSQC
Unk 1	9.680	Broad (s)	160.770	-	-
Unk 2	9.342	Broad (s)	-	-	-
Unk 3	8.543	Broad (s)	141.162	-	-
Unk 4	8.459	Singlet	-	-	-
Unk 5	8.274	Singlet	-	-	-
Unk 6	8.179	Singlet	142.680	-	-
Unk 7	7.969	Doublet	142.755	5.977 (d)	-
Unk 8	6.521	Singlet	-	-	-
Unk 9	6..156	Doublet	88.024	4.793	-
Unk 10	5.994	2 peaks (t)?	-	4.385	-
Unk 11	5.977	Doublet	104.142	7.969	-
Unk 12	5.236	Doublet	93.310	3.545/3.529 (dd)	72.727
Unk 13	4.650	Doublet	97.179	3.242	75.207
Unk 14	4.606	Multi-broad	71.516	2.667	43.976
Unk 15	4.414	Broad (d)	85.176	-	-
Unk 16	4.328	Multi-broad	60.717	3.682	67.237
Unk 17	4.305	(dd)	76.167	2.671/2.351 (dd)	43.870
Unk 18	4.283	Multi-broad	66.387	-	-
Unk 19	4.252	Multi-broad	65.933	-	-
Unk 20	4.240	Multi-broad	66.315	-	-

Unk 21	4.181	(dd)	59.433	2.513/2.403 (dd)	-
Unk 22	4.131	Triplet	63.002	-	-
Unk 23	4.021	(dd)	73.451	-	-
Unk 24	3.995	Overlap	43.828	-	-
Unk 25	3.977	Overlap	61.590	-	-
Unk 26	3.968	Overlap	43.828	-	-
Unk 27	3.948	Overlap	67.729	-	-
Unk 28	3.911	Overlap	71.902	-	-
Unk 29	3.891	Overlap	62.186	-	-
Unk 30	3.887	Overlap	67.789	-	-
Unk 31	3.849	Overlap	62.723	-	-
Unk 32	3.848	Overlap	62.127	-	-
Unk 33	3.839	Overlap	72.736	-	-
Unk 34	3.783	Overlap	44.782	-	-
Unk 35	3.771	Overlap	61.880	-	-
Unk 36	3.763	Overlap	73.036	-	-
Unk 37	3.734	Overlap	61.940	-	-
Unk 38	3.681	Overlap	67.249	Unk 16	-
Unk 39	3.680	Overlap	63.344	-	-
Unk 40	3.620	Overlap	63.231	-	-
Unk 41	3.599	Overlap	67.758	-	-
Unk 42	3.542	(dd)	72.727	-	-
Unk 43	3.474	(t) overlap	77.147	-	-

Unk 44	3.409	(q) overlap	70.799	-	-
Unk 45	3.359	(s) or (d)?	50.126	-	-
Unk 46	3.269	(t)?	54.735	-	-
Unk 47	3.247	Broad (s)	75.292	-	-
Unk 48	3.235	Broad (s)	55.254	-	-
Unk 49	3.229	Broad (s)	55.254	-	-
Unk 50	3.123	Multiplet	53.984	-	-
Unk 51	2.739	Broad weak	37.760	-	-
Unk 52	2.722	Broad weak	39.639	-	-
Unk 53	2.669	(dd)	43.908	Unk 58	-
Unk 54	2.509	(m) overlap	26.574	-	-
Unk 55	2.409	(t) or (s) ov	31.057	-	-
Unk 56	2.403	-	35.284	-	-
Unk 57	2.384	(m) overlap	32.296	-	-
Unk 58	2.361	(dd) overlap	43.908	-	-
Unk 59	2.284	Broad (m)	33.107	-	-
Unk 60	2.276	Broad (m)	34.943	-	-
Unk 61	2.176	Triplet	38.785	1.542,1.294	27.075,23.441
Unk 62	1.920	(s) or (d)	24.552	-	-
Unk 63	1.595	Broad (m)	25.694	1.301,0.891	-
Unk 64	1.545	Broad (m)	26.981	2.178,1.303	38.916
Unk 65	1.428	Broad (m)	23.092	-	-
Unk 66	1.400	Broad (m)	17.794	-	-

Unk 67	1.345	Broad (m)	18.585	-	-
Unk 68	1.329	Broad ov	25.878	-	-
Unk 69	1.314	Broad ov	29.531	-	-
Unk 70	1.298	Broad ov	23.404	-	-
Unk 71	1.280	Broad ov	32.663	-	-
Unk 72	1.272	Broad ov	30.769	-	-
Unk 73	1.216	Broad ov	20.169	-	-
Unk 74	1.214	Broad (m)	25.745	-	-
Unk 75	1.172	Multiplet	20.203	-	-
Unk76	0.947	Broad ov	19.378	-	-
Unk77	0.940	Broad ov	23.483	-	-
Unk78	0.921	Broad ov	15.889	-	-
Unk 79	0.900	Broad ov	19.138	-	-
Unk 80	0.880	Broad ov	22.046	-	-
Unk 81	0.877	Broad ov	11.373	-	-
Unk 82	0.873	Broad ov	14.812	-	-

Key. (s) singlet, (d) doublet, (t) triplet, (q) quartet, (m) multiplet, (dd) double doublet, (ov) overlap and (?) inconclusive assignment.

4.3.3. Statistical analysis of chironomid experiment

The changes to the metabolic pool of the chironomids due to the chemical stressors, fenitrothion, methiocarb and permethrin were investigated. Significant changes to metabolism were identified using PCA, a three dimensional PCA plot of the first three PC's of the original full data set containing all the effects of noise, with only the water peak removed is shown in figure 4.11. Due to the effects of noise the first three PC's were required to show the separation of the treatments. The PC's were then used as descriptor variables in a discriminant analysis, and the plot of the first two discriminant functions (accounting for 84 % and 13 % of the total variation respectively) shows overlapping of the 95 % confidence interval of methiocarb and permethrin groups (fig 4.12). The quality of the supervised classification analysis (LDA) was evaluated using the (mis)classification error rate from the cross validation procedure 'leave one out'. The fenitrothion, methiocarb and permethrin results gave a 100 % correct group classification using all the data and 84 % after the cross validation. The robustness of the classification was tested further using a random permutation test based on 50 repetitions of the LDA using randomised indicator variable for the treatments. This gave an average correct classification of 22 % with none better than 42 %. These low correct classification rates show the robustness of the observed pattern and it would be highly unlikely to occur in this pattern randomly.

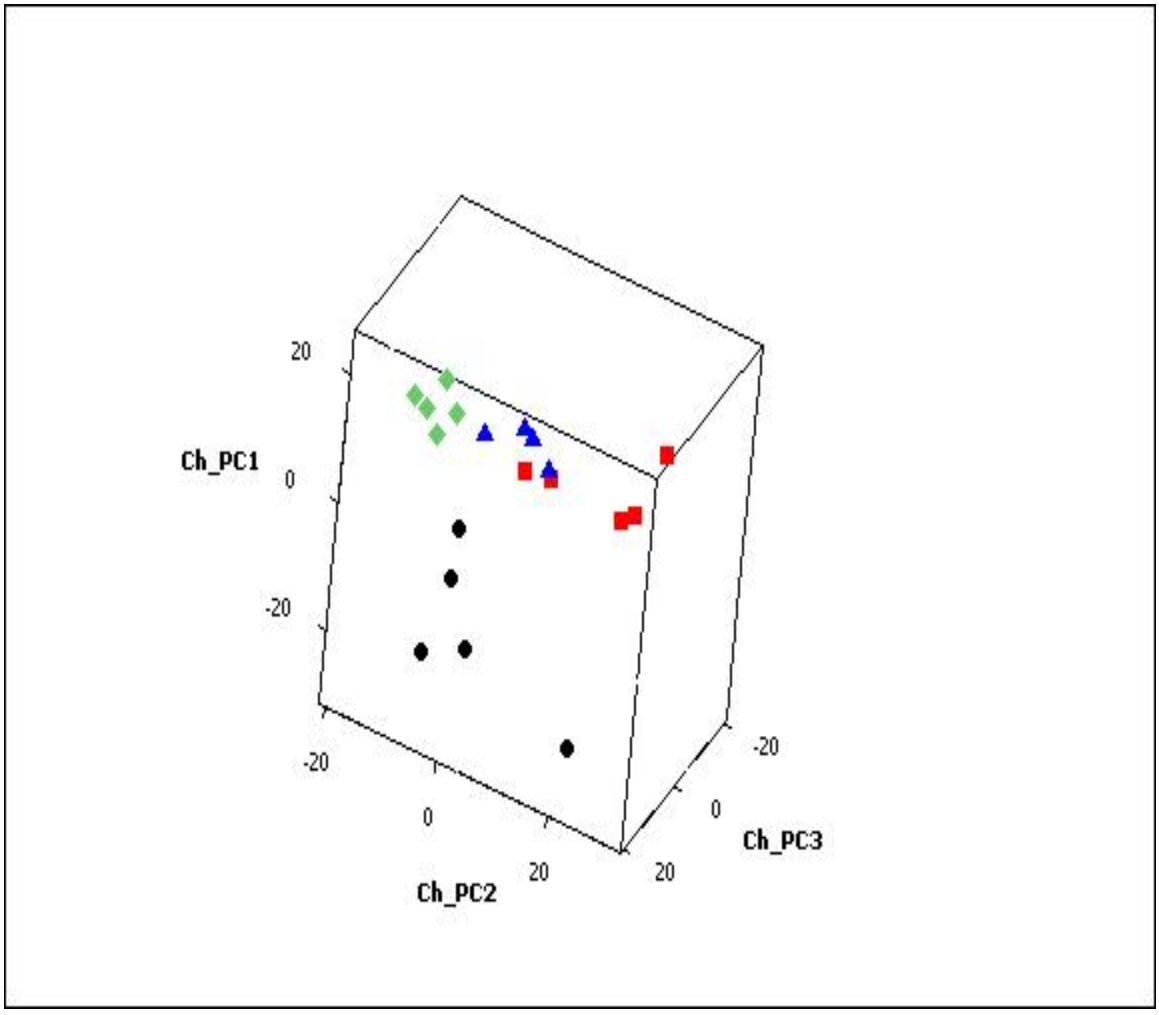

Figure 4.11. A 3-dimensional PCA scores plot showing the group variable separations of the original full chironomid data set of the first three PC's. Black circles-control; red squares-fenitrothion: green diamonds-methiocarb; blue triangles-permethrin.

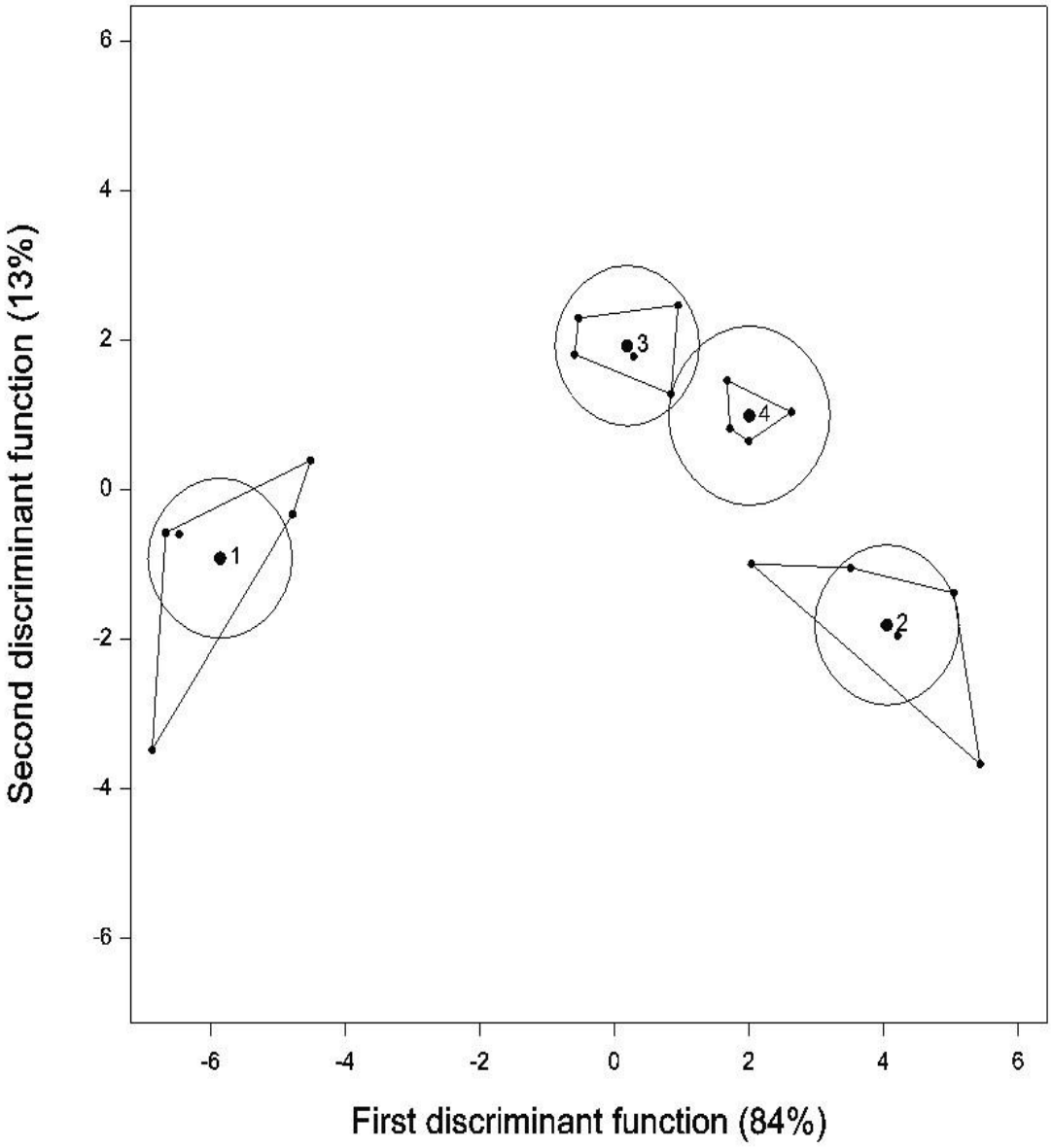

Figure 4.12. The scores on the first two discriminant functions based on the original full chironomid data set. (1 - control; 2 - fenitrothion; 3 – methiocarb; 4 - permethrin). The positions of the treatment means are indicated by the filled circles.

From the subsequent loadings of these PC's further analysis was conducted on those peaks (bins) associated with the highest variability reducing the effect of background noise. These new PC's where then used as descriptor variables in a LDA. The scores on the first five PC's (accounting for 70.3, 15.3, 5.5, 3.4 and 2.9 % of the variation) are presented in Table 4.4 and the correlations (the loadings) of the original variables with those scores are presented in Table 4.5. A LDA was performed using the first three principal components that accounted for 91 % of the variation, and the second discriminant function (2 %) was plotted against the first discriminant function (98 %) (Figure 4.13). This is effectively a single dimension plot since 98 % of the variation is in the first discriminant function.

Table. 4.4. The scores on the first five principal components (accounting for 97.5% of the variation) based on the 24 bins with an association with the treatment.

Sample	PC1	PC2	PC3	PC4	PC5
1	-7.568	-0.703	-1.323	2.198	1.071
2	-7.563	-0.400	-0.755	-0.370	0.615
3	-7.238	0.268	0.932	-2.714	1.026
4	-5.678	1.803	0.297	-0.204	-2.792
5	-6.007	1.714	1.255	1.162	-0.308
6	1.564	-2.819	0.540	0.407	-0.593
7	2.067	-2.372	0.722	-0.215	-0.251
8	1.508	-2.512	0.453	-0.008	-0.615
9	1.912	-2.398	0.459	-0.217	-0.395
10	0.397	-4.574	-0.268	-0.136	0.223
11	3.418	1.877	1.284	0.294	0.145
12	2.305	0.706	0.595	0.786	0.176
13	2.404	0.869	0.902	0.436	0.520
14	2.545	0.976	0.926	0.196	1.032
15	3.235	2.162	1.751	-0.027	0.477
16	2.222	0.774	-1.830	-0.340	-0.108
17	2.279	0.999	-1.791	-0.095	-0.252
18	2.893	1.317	-1.444	-0.191	-0.090
19	2.421	1.004	-1.392	-0.733	0.150
20	2.886	1.309	-1.313	-0.230	-0.032

Table 4.5. Loadings of the original variables on the first three PCs based on 24 bins with an association with the treatment.

Metabolite	PC1	PC2	PC3
Alanine	0.00160	-0.50011	-0.05386
Arginine	-0.23640	-0.05831	-0.07837
Asparagine	-0.23691	-0.03750	-0.05102
Aspartate	-0.20063	-0.18024	-0.05976
Lysine	-0.16014	0.02978	-0.58006
Phenylalanine	-0.23614	-0.01686	0.18623
Tyrosine	-0.20863	-0.13385	0.35618
Unknown 1	-0.23656	0.00118	-0.07773
Unknown 2	-0.24068	0.05336	0.05239
Unknown 3	-0.03290	-0.47607	0.22591
Unknown 4	-0.14208	-0.28489	-0.30327
Unknown 5	-0.21791	0.19264	0.02238
Unknown 6	-0.23081	0.11338	-0.03797
Unknown 7	-0.19121	-0.21481	0.04676
Unknown 8	-0.23575	0.07837	0.03954
Unknown 9	-0.23341	0.08962	0.00790
Unknown 10	-0.23436	0.08880	0.02551
Unknown 11	-0.22915	-0.10476	-0.07557
Unknown 12	-0.14007	-0.29493	-0.35670
Unknown 13	-0.20257	0.07902	0.12867
Unknown 14	0.19987	-0.17676	0.10990
Valine	-0.22896	-0.02083	0.26941
Lactate	0.16631	-0.35889	0.14464

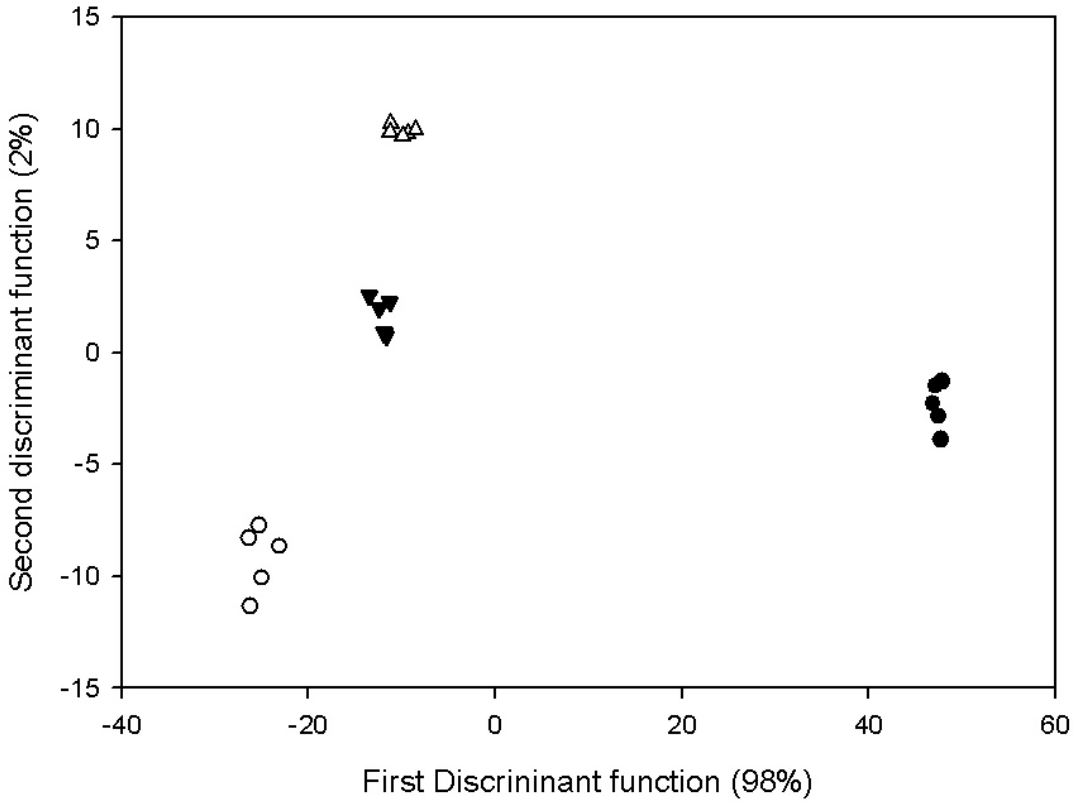

Figure 4.13. A plot of the scores on the second discriminant function on those of the first discriminant function obtained from a linear discriminant analysis based on the scores on the first three principal components of a data set containing all of the metabolites. Each of the treatments controls (filled circles), fenitrothion treated (open circles), methiocarb treated (inverted closed triangles), and permethrin treated (open triangles) is based on five samples of ten 4th instar larvae of *C. riparius*.

Since there were far more variables (24) than observations (20) and there were a number of high correlations between the various metabolites, a subset of nine metabolites and six unknowns were selected on the basis of reliability of the integration relative to the base line, and confidence in the assignment (Table 4.5). A LDA was affected using this subset, and the scores on the first two discriminant functions (accounting for 81% and 19% of the total variation) are plotted in Figure 4.14. The pattern of the four treatment groups based on this sub-set of metabolites is consistent with that obtained for the PCA's based on all of the metabolites identified as having some association with the treatments. The loadings of the subset of metabolites on the

discriminant functions are presented in Table 4.7. These were used to interpret the data.

The correlations between the metabolites indicate the complexity of this data set, and the difficulty of interpreting the data using pairwise comparisons. High correlations between some of the unknowns could indicate that the peaks are associated with the same metabolite. PCA provides a method of dealing with highly correlated variables and produces a series of new uncorrelated variables (the PC's) that contain the original information in the data, and indicate the number of true dimensions in the data set. In this study the first three principal components accounted for 91% of the total variation. However, it would be difficult to interpret these components in terms of the treatments applied because of the large number of variables concerned. Since this data set contained only relatively few dimensions, using a large number of variables would over-define any model produced. Under these circumstances a reduced number of metabolites were selected as described above.

The effects of the treatments on these metabolites were further analysed using an ANOVA of the original variables (or transformed variables where transformation was found necessary (on the basis of Box-cox plots) to render the variance homogeneous. This data was used to identify the fold-change of the metabolite concentration (peak area) from that of the control and in which direction (increase/decrease) with the results in table 4.6. The quality of the supervised classification analysis (LDA) was conducted to analyse the (mis)classification error rate from the cross validation procedure 'leave one out'. The results gave a 100 % correct group classification using all the data and after the cross validation. Once the permutation tests were completed 50 times the

average correct classification rate was 21 % with 96 % of tests resulting in a classification rate of 35 % or less and only two reached 40 % correct. These low percentages show the robustness of this data set as it would be highly unlikely to occur in this pattern randomly.

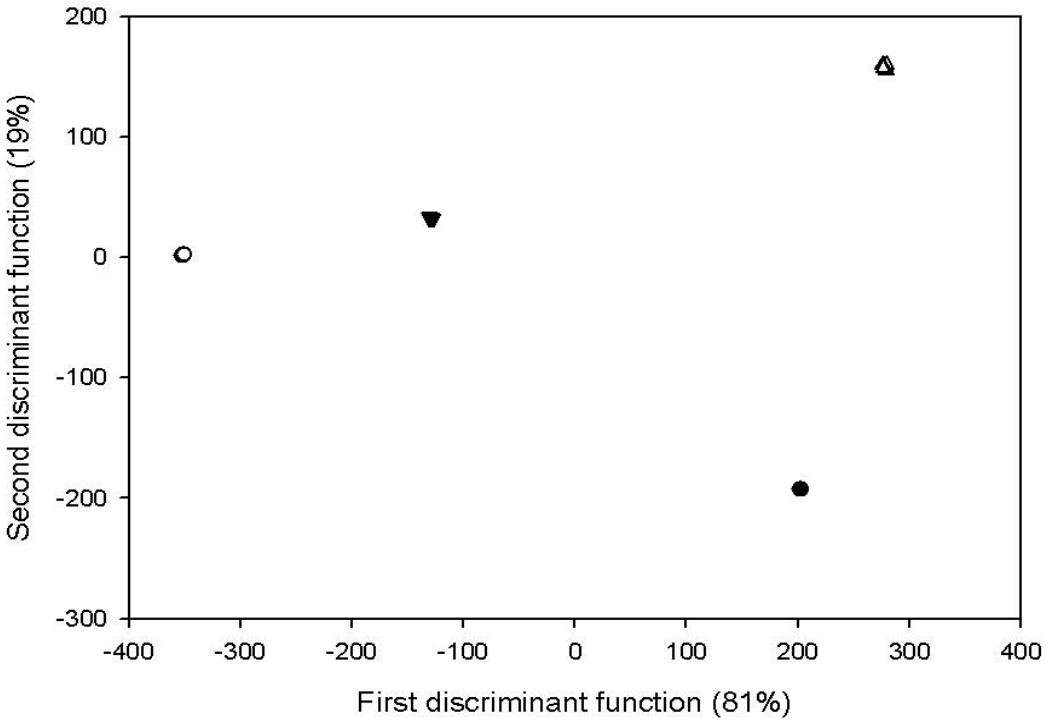

Figure 4.14. A plot of the scores on the second discriminant function on those of the first discriminant function obtained from a linear discriminant analysis based on the peak areas from ^{1}H NMR spectra for nine amino acids. Each of the treatments controls (filled circles), fenitrothion treated (open circles), methiocarb treated (inverted closed triangles), and permethrin treated (open triangles)] is based on five samples of ten 4th instar larvae of *C. riparius*.

Table 4.6. Correlation matrix of the 24 bins with associations with the treatment.

Alanine	1.00				
Arginine	0.72	1.00			
Asparagine	0.69	0.61	1.00		
Aspartate	0.64	0.36	0.22	1.00	
Isoleucine	0.02	0.35	-0.07	-0.10	1.00
Lysine	0.85	0.82	0.65	0.37	0.23
Phenylalanine	0.46	0.49	0.04	0.25	0.39
Tyrosine	0.43	0.14	-0.13	0.70	0.30
Unknown 2	0.35	0.03	0.20	0.61	0.09
Unknown 3	0.31	0.11	0.31	0.36	-0.08
Unknown 4	0.71	0.40	0.58	0.78	-0.04
Unknown 5	-0.48	-0.77	-0.44	-0.20	-0.14
Unknown 6	0.08	-0.10	-0.08	0.64	-0.17
Unknown 7	0.64	0.56	0.12	0.58	0.07
Valine	0.16	0.45	-0.06	-0.09	0.86
Lactate	0.01	-0.10	0.22	0.01	-0.10
	Alanine	Arginine	Asparagine	Aspartate	Isoleucine
Lysine	1.00				
Phenylalanine	0.68	1.00			
Tyrosine	0.24	0.37	1.00		
Unknown 2	0.21	-0.02	0.51	1.00	
Unknown 3	0.35	-0.00	0.26	0.33	1.00

Unknown 4	0.50	0.03	0.48	0.84	0.47
Unknown 5	-0.59	-0.51	0.15	0.08	0.36
Unknown 6	-0.22	-0.19	0.48	0.31	0.47
Unknown 7	0.57	0.76	0.51	-0.11	0.04
Valine	0.34	0.49	0.36	-0.22	-0.02
Lactate	-0.11	-0.63	0.15	0.37	0.33
	Lysine	Phenylalanine	Tyrosine	Unknown 2	Unknown 3
Unknown 4	1.00				
Unknown 5	-0.17	1.00			
Unknown 6	0.39	0.36	1.00		
Unknown 7	0.18	-0.51	0.14	1.00	
Valine	-0.17	-0.14	-0.14	0.33	1.00
Lactate	0.36	0.43	0.12	-0.50	-0.14
	Unknown 4	Unknown 5	Unknown 6	Unknown 7	Valine
Lactate	1.00				
	Lactate				

Table 4.7. Correlations (loadings) of the original variables with the first and second discriminant functions.

Metabolite	1st Function	2nd Function
Alanine	-0.0833	0.0476
Arginine	0.0080	-0.1801
Asparagine	0.0072	-0.1713
Aspartate	-0.0035	-0.0564
Lysine	0.0517	-0.0573
Phenylalanine	-0.0049	-0.3508
Tyrosine	-0.0300	-0.1792
Valine	-0.0143	-0.3115
Lactate	-0.1351	0.2373

Table 4.8. Table of metabolic changes of fenitrothion, methiocarb and permethrin showing the fold-change from the control and p-value from the ANOVA. The arrows indicate whether an increase (↑) or decrease (↓) was identifiedand (NS) not significant.

Metabolite	Methiocarb Fold change	Permethrin Fold change	Fenitrotion Fold change	p-value
Alanine	↑ 1.2	↑ 1.8	↑ 6.9	0.000
Arganine	↓ 1.6	↓ 1.3	↓ 1.1	0.000
Asparagine	NS	↑ 1.3	↑ 1.6	0.520
Aspartate	↑ 7.0	↑ 8.6	↑ 9.8	0.000
Glutamine	NS	↑ 2.9	↑ 1.8	0.000
Isoleucine	NS	↓ 1.4	NS	0.592
Leucine	↓ 2.9	↓ 3.1	↓ 1.3	0.014
Lysine	↓ 2.4	↑ 1.3	↓ 1.1	0.001
Phenylalanine	↓ 3.4	↓ 3.7	↓ 1.5	0.000
Threonine	↑ 1.3	↓ 1.3	↑ 4.1	0.000
Tyrosine	↓ 1.8	↓ 2.2	↓ 1.1	0.000
Valine	↓ 1.1	↓ 1.2	↑ 1.4	0.002

4.3.4. Metabolic effects of exposure to fenitrothion

Exposure of the chironomid larvae to a low concentration (1 μg L^{-1}) fenitrothion lead to significant changes in their metabolism. The scores plot (Figure 4.13) from the LDA shows a clear separation from the controls along the first discriminant function (81 % of the variation). From the correlations of the original variables with the LDA axis, exposure of fenitrothion is associated with a significant increase in alanine and lactate. From the amino acid addition experiments the average increase in alanine from that of the controls (16.4 μM) was 96.3 μM. A standard of lactate was not available for fortification, therefore no concentration data was obtained. However, from the loadings on the first and second discriminant functions (Table 4.5 and Fig. 4.13) it can be seen that increases in both lactate and alanine are associated with exposure to the pesticide.

This pattern of increase of these two metabolites following exposure of chironomid larvae to fenitrothion has previously been reported by (Forcella *et al*., 2007). These authors reported that an increase in alanine is a universal response to stress in many plants and animals. An increase in the level of alanine was also found in the metabolomic study of exposure of *Mytilus edulis* to lindane (section 3.3.5). (Nath *et al*., 1997) suggested that organophosphorous compounds enhance protein metabolism in invertebrates under stress to increase free amino acids available for catabolism. This was partially supported by (Choi *et al*., 2001) who observed an initial increase in protein production (151 %) after 24 h, but a subsequent decrease to 62.7 % of the control after 72 h of exposure to the pesticide. It would therefore be interesting to follow up this study and extend the exposure period to see if the alanine increase continued after 24 h or falls in a similar pattern with the protein content as observed by Choi and co-workers.

4.3.5. Metabolic effects of exposure to methiocarb

The carbamate insecticide, methiocarb, has a similar mode of action to that of fenitrothion as both inhibit acetylcholinesterase (AchE) in the post synaptic membranes in the nervous system. With the OP the inactivation is effectively permanent as it bonds covalently to the active site, whilst with the carbamates AchE can be reactivated by hydrolysis (Hassall, 1990). Despite the similarity in the mode of action of these two insecticides, differences in their impacts on the metabolism of chironomid larvae were detected in this metabolomic study. The score plot (Fig. 4.14) from the LDA again shows a clear separation from the controls along the first discriminant function (81 % of variation). From the correlations of the original variables with the second LDA axis (19% of the variation) (Fig. 4.13 and Table 4.6) it can be seen that exposure to methiocarb is

associated with a changes in concentrations of a number of metabolites relative to the controls. Decreases in arginine/phosphoarginine (from 75 μM to 45.9 μM), leucine (from 24.8 μM to 10.46 μM), and lysine (from 51.3 μM to 27.2 μM) were associated with the exposure to the pesticide relative to the controls. These changes are consistent with the loadings on the second discriminant function (Table 4.4). No significant increase in alanine was observed (16.4 μM control, 15.8 μM treated) separating the carbamate from the control; however, the loadings on the second discriminant function (19% of the variation) indicate that the separation between the pesticide treatments and control is associated with an increase in alanine and lactate, and a decrease in the other amino acids used in the LDA. Forcella *et al*. (2007) investigated the carbamates, carbofuran and carbaryl, and also found no significant increase in alanine, and a decrease in lactate with carbaryl treatment. Our data suggests that lactate increased with exposure, but it is difficult to have confidence in this due to interference by a broad peak associated with residual macromolecules this area, making the integration of the peaks difficult. Forcella *et al*. also noted an increase in aspartate following exposure to carbaryl, but this was not observed in animals exposed to carbofuran, or in the present study following exposure to methiocarb. Methiocarb at relatively high concentrations (< 60 μg L^{-1}) has been shown to reduce the growth of 4^{th} instar larvae. In contrast no significant reduction in growth was found at 10 μg L^{-1} by other workers (Pery *et al*., 2003a, Pery *et al*., 2004). This concentration was 10 times the value used in this study, and this demonstrates shows the increased sensitivity of the biochemical approach to xenobiotic stress.

4.3.6. Metabolic effect of exposure to permethrin

The NOLL for permethrin was 0.1 μg L^{-1}, a factor of ten lower than those for the other two insecticides investigated in this study. From their studies, (Conrad et al., 1999) have suggested that no effects were observed at a concentration of 1 μg L^{-1}. The LC_{50} value found during this study (0.27 μg L^{-1}) was half that of the LC_{50} at 48 h exposure of Conrad's group. This may be attributable to the fact that their group used a formulation 'Picket' where we used a pure compound. Despite the low concentration used, it was still possible to differentiate the permethrin treated population from both the controls and the other treated groups. The score plot (Fig. 4.14) from the LDA again shows a clear separation from the controls along both the first (81% of the variation) and second (19% of the variation) discriminant functions, and from the two other pesticide treatments along the second function. This is attributed to a small increase in alanine (16.4 μM to 29.6 μM), a reduction in valine (6.6 μM to 2.8 μM), phenylalanine (13.4 μM to 6.6 μM), and tyrosine (12.55 μM to 6.5 μM) in the permethrin treated larvae relative to the controls. This is consistent with the loadings on the second discriminant function that also indicate that an increase in lactate relative to the controls is associated with exposure to permethrin. There was a large increase in glutamine (151.7 μM control to 424 μM treatment) but little confidence could be placed on this result since no glutamate was present, and in the period between sample production and the amino acid fortification experiments it is likely that the glutamate originally present had become transformed to glutamine. It was therefore considered unsound to use these results since the final glutamine concentration would represent the pooled glutamine and glutamate in the original sample.

4.4. Metabolite concentration calculations

The concentration of fifteen metabolites was calculated from the intensity of metabolite peaks (Table 4.2). The intensity was used as the broad peaks in the chironomid spectra would interfere with integral values due to the large area found at the base of those peaks. The proton species of the metabolites that gave the clearest peak resolution were chosen (Table 4.9). To be able to use the intensity accurately it was important to spend time shimming each sample to give a consistent resolution between the entire set of samples. The resolution of the TMSP peak was therefore resolved to below 1 Hz. Samples then had a known weighed dose of the metabolites (Table 4.8) added and the resultant intensities recorded. It was then possible, by using the difference in intensity between the additions and the original peak intensities to calculate the ratio change. From these ratios the metabolite concentration could be calculated. Those peaks that had background interference (isoleucine, leucine, valine and alanine) from the broad peak had their intensity values taken from the base of the sharp peak to the top. This eliminated the interference from the broad peak that overlaped. These calculations were then subsequently cross referenced using the same technique using the integral data. A large error with those peaks mentioned above was expected due to the overlapping problems. From this we were able to correlate both sets of data to confirm consistency across the metabolites investigated.

Table 4.9. List of positively confirmed amino acid concentrations between treatments and the proton used for the measurement.

Amino acid	Proton species	Con (Mol/kg)	Met (Mol/kg)	Per (Mol/kg)	Fen (Mol/kg)	Con (μg/mL)	Met (μg/mL)	Per (μg/mL)	Fen (μg/mL)	Addition (μg)
Alanine	hb	1.64	1.58	2.96	11.27	2.3	1.5	1.2	1.8	25
Arginine	hg1	7.50	4.59	5.97	6.78	2.3	1.0	1.1	2.0	60
Asparagine	hb1	1.90	1.42	2.31	2.80	1.5	1.4	2.6	10.1	36
Aspartate	hb1	0.19	1.30	1.22	1.31	8.7	8.0	10.1	11.9	25
Glutamine	hg	15.17	16.60	42.40	25.88	1.6	2.3	1.6	10.8	15
Glutamate	hg	0.0	0.0	0.0	0.0	0.8	0.5	0.5	1.1	35
Isoleucine	hg2	0.83	0.45	0.48	1.12	1.1	0.9	0.7	1.5	28
Leucine	hd2	2.48	1.05	0.95	2.01	3.2	1.4	1.3	2.7	26
Lysine	he	5.13	2.72	7.88	5.30	0	0	0	0	16
Methionine	he	0.0	0.0	0.0	0.0	0.2	1.6	1.9	2.1	18
Phenylalanine	he	1.34	0.64	0.66	1.19	2.2	1.7	2.7	3.3	75
Threonine	hg	1.27	1.92	1.34	9.06	21.8	24.2	61.4	37.4	35
Tryptophan	he3	Low	Low	Low	Low	8.5	4.5	13.1	8.6	16
Tyrosine	he	1.26	0.81	0.65	1.37	0	0	0	0	25
Valine	hg1	0.66	0.45	0.28	0.64	1.0	0.5	0.5	0.5	35

4.5. Conclusions

This novel study was able to show a clear separation between all of the treatment groups and the controls. This demonstrates that this metabolomics technique was able to distinguish between the two pesticides fenitrothion and methiocarb that have a similar mode of action as both are shown to be inhibitors of acetylcholine esterase. The increase in alanine and lactate found in the exposure to fenitrothion corresponds with previous published data on this compound. As the test organisms were exposed to NOLL levels of the toxicants the sensitivity of this profiling method is clearly demonstrated. With the use of one- and two-dimensional NMR spectroscopy we were also able to quantify the metabolic changes in this species of the amino acids identified.

The use of *Chironomus riparius* larvae to investigate water and sediment quality is well established within the regulatory bodies. The utility of the metabolomics approach to understanding the mechanisms of action of model compounds using a simple extraction

and robust analysis technique, demonstrates the potential for the identification and quantification of potential biomarkers in further important contaminants where the mode of action is poorly understood.

Chapter 5

Discussion and Conclusions

5. Discussion

5.1. Method development

5.1.1. Sample preparation

Metabolomics was developed for medical applications, but has been used for a wide range of purposes (e.g., identifying the consequences of genetic modification of organisms, of diseases and parasitic infections, and toxic insults) in a range of organisms, including microorganisms (Dunn and Ellis, 2005). In field of environmental toxicology, it has been used to identify biochemical responses of an organism to external stressors (e.g., starvation, anoxia, exposure to toxicants, including pesticides, industrial chemicals and metals) and can be used to identify potential biomarkers of this stress. Whereas in medical applications and associated animal studies it is possible to obtain large and repeated samples of body fluids from a single organism in order to monitor the time course of biochemical changes following application of a treatment, this is not possible for most invertebrates. Further in clinical studies the samples of body fluids may be analysed directly or after removal of unwanted proteins by precipitation or filtration (Tiziani et al., 2008, Daykin et al., 2002). In studies in invertebrates it is a challenge to obtain a representative sample with sufficient material for analysis, and sample preparation is more difficult. It is often necessary to pool a number of individuals from small species, and whilst this provides an estimate of the average metabolic profile, information on variation between individuals is lost, and extreme (from the tails of the population distribution) metabolic profiles will not be seen.

The challenge in sample preparation for NMR analysis is to obtain a representative extract that is free from compounds (e.g., proteins and lipids) that will produce signals that hide those of the smaller metabolites. The resulting sample should, however, still contain sufficient small molecules at detectable concentrations to provide a representative metabolic profile. In the current study two contrasting organisms, a bivalve mollusc, and an aquatic insect larva, were used. In one, the mussel *Mytilus edulis* , it was possible to sample a specific tissue, the foot, which in contrast with tissues containing regions of the digestive tract was free from contamination with external material and micro-organisms. Here lyophilisation and an acetonitrile based extraction were used, and clear NMR spectra were obtained from a relatively simple procedure. Although there were some sample losses during the lyophilisation process and a limitation of the extractable metabolites the process was quick, simple and reproducible. This was consistent with the findings of (Lin et al., 2007) who evaluated a range of metabolite extraction strategies, and concluded that this approach provided acceptable yield of many common metabolites. However, those workers found some interference from lipids and macromolecules. A similar approach was applied to the preparation of samples of larvae of *Chironomus riparius*. However, because of the small size of this organism (a few mg) it was necessary to combine ten individuals and to extract the whole organisms. Despite the presence of a sclerotized cuticle, and large amounts of haemoglobin, this approach produced clear samples for analysis.

One problem where large samples from an individual are not available is to check the reproducibility and robustness of the sample preparation technique. In this case it is necessary to use sufficient numbers of replicates during method development to obtain measures of variability, and to demonstrate the robustness of the procedure. This is an

important step to provide confidence that the observed variation is due mainly to the biochemical changes induced by the stressor and not from these preparatory steps (Defernez and Colquhoun, 2003). This step is time consuming and may be limited by the number of samples/organisms' to be included in every investigation and is rarely noted as a part of standardised testing for metabolomic studies.

5.1.2. Statistical analysis

The data produced in metabolomic studies is of high dimension, and it is necessary to use data handling techniques that can allow the identification of patterns, and interpretation of the metabolic profiles produced. The use of binning to divide the NMR spectrum into a series of arbitrary segments produces a large non-square data matrix that contains both noise and useful information. Principal component analysis (PCA) is commonly used to reduce the dimensionality of the data set by producing a set of orthogonal eigenvectors (the principal components (PCs)) from the original variables. These do not modify the relative positions of the original observations in the multidimensional space, and still contain a mixture of signal and noise. Further, the PCs are sensitive to the size of the original variables, and it is common practice to use some method to ensure that the original variables (the integrams from the various bins) fall within the same range of order of magnitude. Some sort of normalisation procedure is commonly used. In the current work this was achieved by deriving the PCs from the correlation rather than the variance-covariance matrix. This has the advantage of being unbiased. Many compounds produce peaks in a number of regions of the NMR spectrum, and therefore it might be expected that there are high correlations between

the variables, and so there will be many PCs that are very small. Since the first few PCs contain most of the variation in the data set, then in the current study only the first five PCs were used. In order to reduce the noise, only those bins that were highly correlated with this subset of PCs were used in subsequent analysis. This step was particularly important because the data produced for both species were from relatively small sample populations because of practical limitations (limited space for maintaining the mussels, and the difficulty of rearing large numbers of the chironomid larvae). The use of these small sample sizes (a common problem in environmental toxicity studies) in effect reduces the ability to distinguish real effects either in analyses of variance for single metabolites (where the number of error degrees of freedom is low) , or in linear discriminant analyses (LDA) of sets of variables. In the current study interference was reduced by using the loadings of the original variables on the PCs to identify the bins associated with peaks that are associated with factors that represent large proportions of the total variation, and hence likely to be biochemically important. LDA has been shown as a powerful tool for investigating metabolomics data (Yuan et al., 2008), and optimises the separation between sets of objects (in this case extracts) in identified dimensions (between treatments). It is commonly used with the PCs that have been identified to be of importance. However, the PCs still contain noise, and in the current study this was reduced further by using individual peaks within bins identified as important. Using the PCs as descriptor variables has advantages since by definition they are uncorrelated. This is not the case for the individual peaks in the NMR spectrum. These can be correlated because either (where it is not possible to assign them) they are both part of the signal from the same compound, or because in metabolism a change in one metabolite is linked with changes in another (e.g., one might be substrate that is converted to the other). If LDA is to be used with the

individual peaks, then it is necessary to examine the correlation matrix of the variables that could potentially be used, and omit one of each pair of highly correlated variables from the analysis. This will reduce redundancy, and reduce over-definition of the discriminant function. A further problem with LDA is that it assumes normality and the same within group covariance matrix for all samples. Violation of these assumptions may not reduce the ability to achieve a separation between groups, but may negate the use of simple tests of the significance of differences between samples (Manly, 1994). It is therefore advisable to use randomisation tests to assess the significance of observed separations.

5.1.3. Fortification experiments

One area of this study where the non-destructive nature of NMR became a distinct advantage was that of the fortification experiments. The addition of known amounts of pure standard amino acids to the samples greatly increases confidence in the assignment of the peak data for each metabolite. This removes the problems of overlapping peaks within the data set as all of the associated peaks of a particular metabolite increases with the addition. Therefore metabolites with similar structures can be distinguished from one another. These additions also aided in the quantification of the identified metabolites with use of the two dimensional experiments leading to accurate quantification. This type of experiment is not often reported as being utilised in metabolomic experiments and is probably due to the time consuming nature of the process. The metabolites also have to be readily available with some idea of their occurrence within the samples known. Throughout all the experiments conducted there

remained many unidentified peaks that were labelled of interest to the metabolic perturbations, but could not be formally identified. The utilisation of further dimensions of NMR analysis may increase the identification process as other nuclei of interest that are compatible could be investigated (Baverel et al., 2003, Fan, 1996, Grottum et al., 1998, Viant et al., 2001).

5.2. Toxicology

5.2.1. Toxicological assays

Many pesticides are found in natural waters; generally in low concentrations, but with seasonal peaks corresponding with times of field applications, and subsequent rain events that can cause run off from fields into water courses. It is therefore preferable to use environmentally relevant concentrations in laboratory exposure trials. This study investigated the effects of the toxicants at low chronic concentrations. However, there are difficulties associated with using low, environmentally realistic concentrations. A large uncertainty is associated with the estimates of toxic concentrations in the tails of the tolerance distribution and this makes it difficult to estimate the no effect concentrations that are used in setting environmental quality standards, and that were used in this study. In order to compare the impacts of different toxicants it is necessary to use equitoxic concentrations. This is not straightforward to achieve, and it would be useful to extend the range of doses used, and to increase the replication in order to achieve reliable estimates of the no effect concentration. In parallel with this it would be helpful to carry out the metabolomic studies over a wider range of concentrations in

order to obtain dose-response relationships for the different classes of insecticide. It would then be possible to see whether similar metabolite profiles are observed in the different classes of insecticides for equitoxic doses. However, this would require large numbers of animals and many hours of NMR time. Further it would not be possible to use higher doses since most of the treated animals would become moribund, and could not be used.

5.2.2. Modes of action

Many non-polar molecules display a general toxicity as they accumulate within biological membranes and disrupt metabolic processes in a non-specific way (Buchwalter *et al*., 2004, Hong *et al*., 2004, Lopez, 2003, Mordaunt *et al.*, 2005). Others may have more specific effects by interacting with some component of the biological regulatory system (e.g., inhibiting an enzyme, or acting as an agonist or antagonist of a neurotransmitter or hormone. For some of these compounds where there is a well defined mode of action, and a readily measured indicator of poisoning it is possible identify biomarkers. The organophosphate and carbamate insecticides fall within this category; they both inhibit acetylcholine esterase at cholinergic synapses, and the activity of this enzyme provides a directly relevant biomarker. However, for some compounds such as the pyrethroid insecticides that act as axonal poisons by disrupting ion channel (primarily the voltage-gated sodium channel) functioning, there is no obvious biomarker, and toxicologists use behavioural and neurophysiological measurements as indicators of poisoning. There are similar problems in detecting exposure to lindane.

Atrazine was designed to disrupt the photosynthetic pathways in plants, and inhibits the Hill reaction and its associated non-cyclic photophosphorylation (Shimbakuro and Swanson, 1969). However, it is known to have some toxicity in vertebrates, and some less well defined toxicity in invertebrates. In the rat, atrazine disrupts the ovulatory surge of luteinising hormone by disrupting the hypothalamic regulation of the pituitary, and through this inducing premature reproductive ageing and increased incidence of mammary tumours (Cooper et al., 2007). However, these effects are not seen in other species. In invertebrates the effects were diverse and generally produced only at high environmental concentrations (Eisler, 1989). A recent proteomic analysis of atrazine toxicity in adult Drosophila found that some 28 proteins associated with energy production were differentially expressed between treated and control animals, indicating a role in affecting mitochondrial electron transport and oxidative stress (Thornton et al., 2010). This is consistent with results presented in section 3.5.7.of this thesis. For compounds such as atrazine where there is no well defined mode of action in many animal species, then metabolomics can utilised as a tool to provide some insight. It is important, however, to differentiate between the specific effects of toxicants and more general environmental stress effects. The use of well defined controls is essential.

It is extremely difficult to gain an understanding of these mechanisms of toxicity using traditional toxicological endpoints. Modern rapid enzymatic bioassays that provide measurements of the activity of individual enzymes can provide baseline data for normal (unexposed control) activity, and detect changes following exposure of an organism to stress. However, there are many metabolic pathways that can be affected,

and in order to identify the key activities that are affected it would be necessary to use a battery of such assays (Sogorb and Vilanova, 2002, Sturm and Hansen, 1999).

5.2.3. Biomarkers

Two assays of the type described above have been used to investigate effects on mussel populations thought to be affected by sewage pollution. The biomarkers, heat shock protein levels (HSP) and glutathione S-transferase (GST) were investigated, but it was found that low salinity levels also have a major effect on these biomarkers, with feeding, seasonality and reproductive state also contributing to fluctuations (Lyons et al., 2003). The same biomarkers have also been used to evaluate the effects of chemical stressors on *Chironomus* sp. (Park et al., 2009, Park et al., 2010, Planello et al., 2010, Yoshimi et al., 2002). Both species displayed changes in regulation of these biomarkers as an indication of toxicity of the chemical stressor investigated. However, this shows only that a toxicant is having an effect on an organism biochemically, but gives no further information on other metabolic pathways that may be affected. As the metabolomics approach makes no prior assumptions as to the mechanisms involved in a response to a stressor this approach can be a guide to further, more focussed biochemical investigations. This study investigated chemical stressors with similar secondary lesions resulting from disruption of different primary target sites (fenitrothion (OP), methiocarb (carbamate) that are acetylcholine esterase inhibitors and the synthetic pyrethroid, permethrin that is a cation channel disruptor). All three lead to a general uncontrolled increase in levels of nervous activity. With such a general impact on the systems regulating metabolism, then any neurotoxicant causing similar effects in

the nervous system might be expected to produce similar effects on metabolism. In this study it was therefore surprising to find that it was possible to differentiate between the metabolite profiles of chironomid larvae exposed to these three neurotoxicants that produce similar gross symptoms, and where the OP and carbamate share a common primary target site. It may be the use of low concentrations that did not cause mortality or gross symptoms of poisoning that allowed more subtle changes to be detected.

In this study the majority of metabolites identified were amino acids, and these are particularly suitable for NMR spectroscopic analysis due to their high methyl group content. However, they are also particularly important for aquatic species for osmoregulation, where the regulation of solute concentrations across permeable membranes is an important process for animals that use water as a respiratory medium (de Vooys and Geenevasen, 2002). They need to maintain control of the osmotic concentration of their metabolic pool allowing them to regulate the cell volume, and the concentrations of key metabolites. Marine organisms are known to use amino acids as osmolytes, and so toxicants that may modify metabolism may have a negative impact on such processes (Abe, 2002, de Vooys and Geenevasen, 2002). They are also important in some aquatic insect larvae (Edwards, 1982). Therefore changes in amino acid concentrations may provide useful patterns of toxicity and along with other metabolites help to identify useful biomarkers of exposure to a particular chemical stress.

The study using *Mytilus edulis* as a model organism identified patterns of changes in metabolites that were consistent with published physiological and biochemical information on the toxicity of two different pesticides, lindane and atrazine, and to

differentiate changes associated with the latter from changes produced by hypoxia and starvation. Whereas lindane produces an overall depression of metabolic activity, the herbicide produced an overall stimulation of oxidative metabolism. For both compounds the low dose group fell on the trajectory between the control and high dose groups. This indicates the potential utility of using a range of doses and exposure times to define dose-response relationships. This may be of help in a regulatory context where it is often difficult to establish a reliable NOEL.

Despite the success in identifying changes in metabolic profiles that can be used to differentiate the effects of primary lesions caused by exposure to atrazine from background controls, and the effects of environmental stressors, the study did not identify any specific biomarker that could be measured using a rapid biochemical assay. Similar conclusions can be drawn from the study using the chironomid larva as a model organism. As for the routine use of ^{1}H-NMR for screening environmental samples, it may be necessary to combine proteomic and metabolomic methods to identify enzymes associated with particular changes in characteristic of poisoned animals.

5.3. Future work

In order to progress this work it would be important to Identify more metabolites in the spectra obtained from both species. Many of the metabolites that displayed a significant change with the various treatments remained unidentified and could provide important information on the metabolic changes resulting from the toxic insults. These could be more representative of a particular metabolic pathway than those of the amino acids identified during this study.

A reduction in the amount of *Mytilus* tissue used in original samples (100 mg) to ~ 10 mg as used for the chironomids may eliminate the problem of the dynamic range of the metabolites betaine and taurine. This reduction would enable a similar regime of 2 dimensional experiments and subsequent metabolite fortification experiments. This would facilitate metabolite identification and quantification. This would be especially important for the unidentified peaks that are associated with toxic and environmental stressors.

A reduction in the amount of manual input needed per sample by automating sections of the analysis to reduce the time taken from sample preparation to data analysis would be invaluable. The writing of macros within the ACD labs software would allow a set of FID data to be collected and the phasing, baseline correction, referencing and bucket integration steps to be completed quickly and efficiently, and the data as a series to be exported directly into the statistical software and analysed.

Both of the studies reported in this thesis used only a single time point in the poisoning process. It would be helpful to produce a time-dose response for each toxicant in order to gain a better understanding of metabolism changes during the onset of poisoning, and as poisoning progressed with the onset of detoxification and elimination. Useful information could also be obtained by removing the organism from the exposure and following the metabolic recovery.

The mode of action of atrazine in animals is still poorly understood, despite much research. This study indicates that metabolomic methods might be able to provide some basic information. In order to follow the changes in energy metabolism it would be

necessary to follow a time trajectory at a range of doses to establish a time-dose response surface. The use of complementary proteomic measurements, and ^{31}P NMR would provide a data set with a comprehensive description of changes in energy metabolism. Some of the key metabolites of atrazine have been reported to be toxicologically active, and these could be included in the study.

There is scope for widening the study using chironomids to include a range of toxicants of current interest, and these would include some pharmaceuticals that are pseudo persistent in the aquatic environment. This model could also be used to investigate the metabolic impact of mixtures of compounds where synergism and antagonism can occur (Anderson and Zhu, 2004). Organisms in the environment are rarely exposed to a single contaminant this generally occurs only under laboratory conditions, and the problem of estimating the toxicity of mixtures of pollutants is currently of great interest to regulators. Metabolomic methods offer solutions to a number of the problems currently facing industry and regulatory authorities who are using biological information as the basis of environmental quality standards.

5.4. Conclusions

1. A simple solvent precipitation extraction utilising acetonitrile as the co-solvent with deuterated water produced samples that gave clear, well resolved ^{1}H NMR spectra of the extractable metabolic pools of tissue from the two species; *Mytilus edulis* and *Chironomus riparius*. The samples were reproducible and the intrinsic intraspecific variation was estimated within the control groups.

2. The study of *Mytilus edulis* identified a clear differentiation between the control, low and high dose treatment groups for both lindane and atrazine. The latter were well separated from groups subjected to hypoxia and starvation. This was achieved with less than 20 metabolites being formally identified and thus shows the robust nature of this technique.

3. In the *Chironomus riparius* study it was necessary to use whole body homogenates of ten individuals per sample in order to obtain sufficient material to analyse. Groups exposed to three different neurotoxicants toxicants (fenitrothion, methiocarb, and permethrin) that produce similar secondary lesions could be distinguished from each other and from the control group on the basis of 12 metabolites. This reinforces the robustness of the simple extraction technique combined with ^{1}H NMR.

4. The use of PCA enabled the reduction of the dimensionality of the raw data, identification of important variation, and removal of interferences due to noise. Subsequent application of LDA provided robust separations of the various treatment groups.

5. Although this study identified a series of metabolites that could be used to allocate samples to treatment groups, it was not possible to identify individual biomarkers that could be used to identify organisms that had been exposed to a particular toxicant.

Chapter 6

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